

# Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL-21 deficiency

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**Background:** Alterations of immune homeostasis in the gut can result in development of inflammatory bowel disease (IBD). Recently, Mendelian forms of IBD have been discovered, as exemplified by deficiency of IL-10 or its receptor subunits. In addition, other types of primary immunodeficiency disorders might be associated with intestinal inflammation as one of their leading clinical presentations.

**Objective:** We investigated a large consanguineous family with 3 children who presented with early-onset IBD within the first year of life, leading to death in infancy in 2 of them.

**Methods:** Homozygosity mapping combined with exome sequencing was performed to identify the molecular cause of the disorder. Functional experiments were performed to assess the effect of IL-21 on the immune system.

**Results:** A homozygous mutation in *IL21* was discovered that showed perfect segregation with the disease. Deficiency of IL-21 resulted in reduced numbers of circulating CD19<sup>+</sup> B cells, including IgM<sup>+</sup> naive and class-switched IgG memory B cells, with a concomitant increase in transitional B-cell numbers. *In vitro* assays demonstrated that mutant IL-21<sup>Leu49Pro</sup> did not induce signal transducer and activator of transcription 3 phosphorylation and immunoglobulin class-switch recombination.

**Conclusion:** Our study uncovers IL-21 deficiency as a novel cause of early-onset IBD in human subjects accompanied by

defects in B-cell development similar to those found in patients with common variable immunodeficiency. IBD might mask an underlying primary immunodeficiency, as illustrated here with IL-21 deficiency. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

**Key words:** IL-21, early-onset inflammatory bowel disease, common variable immunodeficiency, exome sequencing

A tightly regulated balance of the immune system is critical for immune homeostasis and, if altered, may be responsible for the development of inflammatory bowel disease (IBD).<sup>1</sup> Genome-wide association studies to identify genetic factors of complex IBD traits have suggested that both genetic and environmental factors contribute to the disease.<sup>2-4</sup> The majority of susceptibility loci identified have revealed genes important in immunologic processes.<sup>5</sup> More recently, early-onset Mendelian forms of IBD have been recognized, as illustrated by the discovery of IL-10 (receptor) deficiency.<sup>6,7</sup>

Intriguingly, patients with various primary immunodeficiency disorders can also exhibit intestinal inflammation as one of their leading symptoms.<sup>8</sup> For instance, qualitative or quantitative neutrophil defects, such as chronic granulomatous disease<sup>9</sup> or G6PC3 deficiency,<sup>10</sup> have been associated with chronic intestinal inflammation. In addition, patients with partial T-cell deficiency, Wiskott-Aldrich syndrome,<sup>11,12</sup> LPS-responsive beige-like anchor (LRBA) deficiency,<sup>13</sup> or defects in the development of regulatory T cells<sup>14</sup> might present with colitis. Similarly, primary B-cell deficiencies, including the subgroup of common variable immunodeficiencies (CVIDs), frequently display an IBD-like phenotype.<sup>15,16</sup> These observations indicate that multiple immunopathologic processes can result in IBD and point to the gastrointestinal tract as a particularly vulnerable site for aberrations of immune homeostasis.

We here describe IL-21 deficiency as a novel primary immunodeficiency, which associates a CVID-like B-cell deficiency with early-onset IBD.

## METHODS

### Patients and ethics

This study has been approved by the responsible local ethics committee. Biological material was obtained on informed consent in accordance with the Declaration of Helsinki. The patient was followed up and treated at the Departments of Immunology and Gastroenterology, respectively, at Ankara University in Turkey.

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**Abbreviations used**

|         |                                                  |
|---------|--------------------------------------------------|
| APC:    | Allophycocyanin                                  |
| CD40L:  | CD40 ligand                                      |
| CVID:   | Common variable immunodeficiency                 |
| DMEM:   | Dulbecco modified Eagle medium                   |
| IBD:    | Inflammatory bowel disease                       |
| IL-21R: | IL-21 receptor                                   |
| MD:     | Molecular dynamics                               |
| NK:     | Natural killer                                   |
| PE:     | Phycoerythrin                                    |
| SNP:    | Single nucleotide polymorphism                   |
| SNV:    | Single nucleotide variant                        |
| STAT:   | Signal transducer and activator of transcription |
| TCR:    | T-cell receptor                                  |
| WT:     | Wild-type                                        |

**Flow cytometry–based immunophenotyping**

Flow cytometric analyses were performed on a BD LSR Fortessa, BD FACSCanto, or BD FACSCalibur. In brief, PBMCs from the patient and a healthy control subject were isolated by using Ficoll density gradient centrifugation and stained for 20 minutes at 4°C with mouse anti-human antibodies: CD3–allophycocyanin (APC)–H7 (clone SK7, BD Biosciences, Vienna, Austria), CD4–APC (clone RPA-T4, BD Biosciences), CD8–V500 (clone RPA-T8, BD Biosciences), CD19–PerCPCy5.5 (clone HIB19; eBioscience, Vienna, Austria), CD21–phycoerythrin (PE; clone B-ly4, BD-Biosciences), CD27–Brilliant violet (clone M-T271, BD Biosciences), CD38–PECy7 (clone HIT2, BD Biosciences), IgD–fluorescein isothiocyanate (clone IA6-2, BD-Biosciences), IgM–APC (clone G20-127, BD Biosciences), T-cell receptor (TCR)  $\alpha\beta$  (fluorescein isothiocyanate, clone WT31, BD Biosciences), and TCR $\gamma\delta$  (PE, clone 11F2, BD Biosciences).

Activation of B cells, in-depth B-cell phenotyping, and stimulation were carried out, as published previously.<sup>17–19</sup>

**Genetic analyses**

Genomic DNA sequencing of *IL10*, *IL10RA*, and *IL10RB* was performed according to standard methods (see Table E1 the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) for details). Single nucleotide polymorphism (SNP)–based homozygosity mapping and exome sequencing were performed as described previously<sup>20</sup> with minor modifications (see the Methods section in this article's Online Repository).

**Recombinant protein production**

Recombinant protein production with the HEK-EBNA system was carried out, as described previously.<sup>21,22</sup> In brief, HEK-EBNA cells were seeded in a 10-cm dish in complete Dulbecco modified Eagle medium (DMEM; PAA, Invitrogen, Vienna, Austria) and transfected with 5  $\mu$ g of vectors encoding N-terminally His-tagged IL-21<sup>WT</sup> or IL-21<sup>Leu49Pro</sup>.

Transfected cells were selected with complete DMEM containing 1  $\mu$ g/mL puromycin (Sigma-Aldrich, Vienna, Austria). Once confluence was reached, cells were set on serum-free DMEM to initiate IL-21 production. Supernatants were collected after 1 week, filter sterilized, and frozen at –80°C. Trichloroacetic acid precipitation was performed at –20°C overnight to test successful IL-21 production. Subsequently, proteins were washed with 80% acetone and 100% acetone once each, air-dried, and resuspended in Laemmli buffer. Equal volumes of sterile-filtered supernatants were loaded on a 12% acrylamide gel, and the membrane was probed against the His-tag of the produced proteins to assess equal production and stability of wild-type (WT) and mutant IL-21 protein, respectively. Subsequently, stimulation experiments were carried out with equal volumes of supernatants and thus equal amounts of mutant and WT IL-21, respectively.

**Stimulation of Jurkat cells**

Jurkat cells ( $5 \times 10^6$  per condition) were serum starved to reduce global phosphorylation levels and stimulated with equal volumes of IL-21<sup>WT</sup>, IL-21<sup>Leu49Pro</sup>, or 10 ng/mL commercially available recombinant IL-21 (eBioscience), respectively, for 30 minutes. Cells were washed once with cold PBS and spun down, and the pellet was snap-frozen in liquid nitrogen. Protein was isolated with cell lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100 (pH 7.1), and complete protease inhibitor cocktail (Sigma-Aldrich). Samples were run on an 8% acrylamide gel at a constant voltage of 110 V for 2 hours and blotted at 350 mA for 1.5 hours. Membranes were blocked with 5% BSA–Tris-buffered saline containing 0.05% Tween (Sigma-Aldrich) and incubated with the respective antibodies overnight at 4°C.

**Immunoblot analysis**

The primary antibodies used for Western blot analysis were rabbit anti-human signal transducer and activator of transcription (STAT) 3 and phospho-STAT3 (Tyr701, 58D6; both from Cell Signaling, Frankfurt am Main, Germany). Primary antibodies directed against His (AD1.1.10) and a mouse anti-human glyceraldehyde-3-phosphate dehydrogenase antibody (clone 6C5; both from Santa Cruz Biotechnology, Heidelberg, Germany) were used. Horseradish peroxidase–conjugated goat anti-rabbit (Bio-Rad Laboratories, Vienna, Austria) and goat anti-mouse (BD Biosciences) secondary antibodies were detected by using a chemiluminescent substrate (Amersham ECL Prime Western Blotting Detection Reagent, GE Life Sciences, Vienna, Austria). Detection was performed by using autoradiography with Hyperfilm ECL (Fisher Scientific, Vienna, Austria).

**T-cell proliferation analysis**

T-cell proliferation assays were carried out, as described previously.<sup>20</sup>

**T-cell V $\beta$  spectratyping**

TCR V $\beta$  spectratyping was performed, according to the method of Pannetier et al,<sup>23</sup> with minor modifications (see Table E2), as described previously<sup>20</sup> (see the Methods section in this article's Online Repository).

**In silico analyses of IL-21**

In brief, molecular dynamics (MD) simulations of WT and mutant IL-21 were performed by using the coarse-grained model FREADDY<sup>24</sup> implemented in the MOIL<sup>24,25</sup> molecular modeling package with different degrees of flexibility. All simulations predicted the same qualitative behavior. For details, please see the Methods section in this article's Online Repository.

**RESULTS****Clinical characterization of the patient**

The index patient (currently 11 years of age) was born to healthy Turkish consanguineous parents (first-degree cousins). Two of 8 of the patient's siblings died of severe diarrhea before 1 year of age (2 months and 8 months, respectively). The index patient's disease is characterized by both early-onset IBD and a CVID-like immunodeficiency, as specified below.

**IBD.** The patient initially presented to the hospital at 2 months of age with persistent, mucoid, nonbloody diarrhea and recurrent oral aphthous ulcers. Additionally, he exhibited signs of chronic disease, such as fatigue and failure to thrive, with body weight being constantly less than the third percentile (not shown). He did not show any general skin lesions, perianal lesions, fistulas, uveitis, or signs of arthritis, respectively. During the following years, diarrhea persisted, and both malnutrition and finger clubbing became apparent. Results of microbiologic analyses in

feces for *Cryptosporidium* species, *Giardia lamblia*, rotavirus, and adenovirus were negative. Colonoscopy revealed macroscopically a loss of vascular pattern and mucosal erythema, as well as edema and aphthous ulcers (Fig 1, A). Biopsy specimens showed eosinophil and neutrophil infiltration, focal cryptitis, focal active colitis, and noncaseating granuloma, as seen in patients with typical Crohn disease (Fig 1, B). As a part of a local study protocol for patients with early-onset IBD, magnetic resonance cholangiopancreatography was performed, which did not reveal any abnormalities. The patient was evaluated for familial Mediterranean fever, therefore exons 2 and 10 of Mediterranean fever gene (*MEFV*) were sequenced, and a heterozygous carrier status of a common mutation (p. Met694Val) was detected. On the basis of colonoscopic and histologic findings, the patient was given a diagnosis of Crohn disease. Treatment with mesalazine, omega-3 fatty acids, and supplementary enteral feeding was initiated, but chronic diarrhea persisted. The patient, now 11 years of age, is still malnourished, with body weight persistently less than the third percentile (data not shown).

**Immunodeficiency.** At the age of 4 years, the patient was referred for assessment of a potential underlying immunodeficiency. Laboratory examinations revealed increased IgE levels (119 kU/mL), while IgG levels (338 mg/dL) and isohemagglutinin levels were reduced (titer 1:2) (Table I). In retrospect, the patient experienced recurrent and severe upper and lower respiratory tract infections at an unusually high frequency from the age of 1 year onward. Not all criteria for CVID diagnosis were met as IgG was the sole type of reduced immunoglobulin (Table I), the patient was therefore given a diagnosis of a CVID-like immunodeficiency.<sup>26,27</sup> Intravenous immunoglobulin replacement together with prophylactic trimethoprim-sulfamethoxazole therapy was started. At present, the patient exhibits signs of severe chronic pulmonary infections, such as finger clubbing.

**Detailed immunologic work-up.** The patient was vaccinated according to general recommendations, receiving vaccinations against BCG, hepatitis B virus, diphtheria-pertussis-tetanus-polio, and measles. Anti-hepatitis B virus surface antigen titers were found to be borderline low, and antibody titers against BCG were negative. He was also evaluated for autoantibodies, such as anti-neutrophil antibodies, anti-neutrophil cytoplasmic antibodies, thyroglobulin antibodies, and anti-gliadin IgA and IgG, and results were negative at 9 years of life. PCR-based evaluation for cytomegalovirus and human immunodeficiency virus was negative. CD40, CD40 ligand (CD40L), and forkhead box protein 3 (FOXP3) expression was evaluated by means of flow cytometry to exclude known hereditary immunodeficiency syndromes; results were found to be normal (data not shown). *Cryptosporidium* species in feces was absent at age 6 years and 9 years, respectively. Neutrophil oxidative burst assays at age 5 years to assess potential chronic granulomatous disease did not show any abnormalities (data not shown). Because of the severity and early onset of IBD in this patient, the *IL10*, *IL10RA*, and *IL10RB* genes were sequenced, but no mutation could be detected (data not shown).

### Mutation identification in the *IL21* gene

Given the consanguinity in the family and the occurrence of early-onset IBD in 3 of 8 siblings, a monogenetic defect with

autosomal recessive inheritance was suspected (Fig 1, C). SNP array-based homozygosity mapping (see Tables E3 and E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) and exome sequencing were then performed to identify the underlying genetic defect. Hits from exome sequencing were filtered for homozygous intervals present in the patient and validated by capillary sequencing (see the Methods and Results sections in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). A total of 2 hits which showed perfect segregation among the core family members were validated. No obvious role for the variant in *ENPEP* in the patient's disease could be detected (see the Results section in this article's Online Repository). The second hit, a homozygous mutation in *IL21* (c.T147C, p.Leu49Pro), showed perfect segregation under the assumption of autosomal recessive inheritance with full penetrance and could not be detected in existing SNP databases, such as ENSEMBL, dbSNP, or UCSC (date of accession: December 30, 2013; Fig 1, C and D). In light of the marked IBD phenotype in all 3 affected subjects in this family, we intersected exome data from the patient with known SNPs predisposing for IBD (taken from <http://www.genome.gov>). However, no known SNPs in *IL23R* or *NOD2* associated with IBD could be detected in the patient (data not shown).

The mutated residue IL-21<sup>Leu49</sup> is highly conserved throughout evolution (Fig 1, E). Prediction of the protein-folding stability of the IL-21<sup>Leu49Pro</sup> variant using the CUPSAT protein stability analysis tool<sup>25</sup> suggested that any change in IL-21<sup>Leu49</sup> would reduce the stability of the native state. Notably, the strongest destabilization of the native state of protein folding was predicted by changing IL-21<sup>Leu49</sup> to proline (see Table E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). CUPSAT predictions were supported by using coarse-grained MD simulations of IL-21<sup>WT</sup> and IL-21<sup>Leu49Pro</sup>. In the simulation of IL-21<sup>WT</sup>, IL-21<sup>Leu49</sup> remains fully buried within the protein core, whereas the protein core of the IL-21<sup>Leu49Pro</sup> mutant is predicted to be unstable in the original orientation, undergoing a transition to the outer side of helix A into the gap between helices A and C (Fig 1, F) within 10 ns (Fig 1, G). This transition is accompanied by an extension of the  $\alpha$ -helical part of the helix A (the C-terminal end of the A is a 3<sub>10</sub> helix in the WT fold) and slight modification of the relative position of helices A and C (Fig 1, F). Because the crystal structure of IL-21 bound to the IL-21 receptor (IL-21R; PDB id 3TGX) demonstrates that helices A and C of IL-21 are critical for receptor binding and initiation of signaling of the Janus kinase-Stat signaling cascade,<sup>28</sup> the predicted conformational change caused by the *IL21*<sup>Leu49Pro</sup> mutation most likely decreased the affinity of IL-21 binding to IL-21R.

### Functional consequences of IL-21 deficiency

Assuming that the patient's disease is most likely caused by defective IL-21 function that impairs adaptive immunity,<sup>29,30</sup> a more detailed analysis of the patient's immunologic phenotype was performed. Compared with healthy control subjects, the patient had fewer circulating CD19<sup>+</sup> B cells and almost no CD19<sup>+</sup>CD27<sup>+</sup>IgD<sup>+</sup> marginal zone-like and CD19<sup>+</sup>CD27<sup>+</sup>IgD<sup>-</sup> class-switched memory B cells (Fig 2, A, and see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), resulting in a relative increase in CD19<sup>+</sup>CD38<sup>hi</sup>CD23<sup>-</sup> transitional B-cell numbers (Fig 2, B). Accordingly, very few IgG<sup>+</sup> or IgA<sup>+</sup> cells were detected in

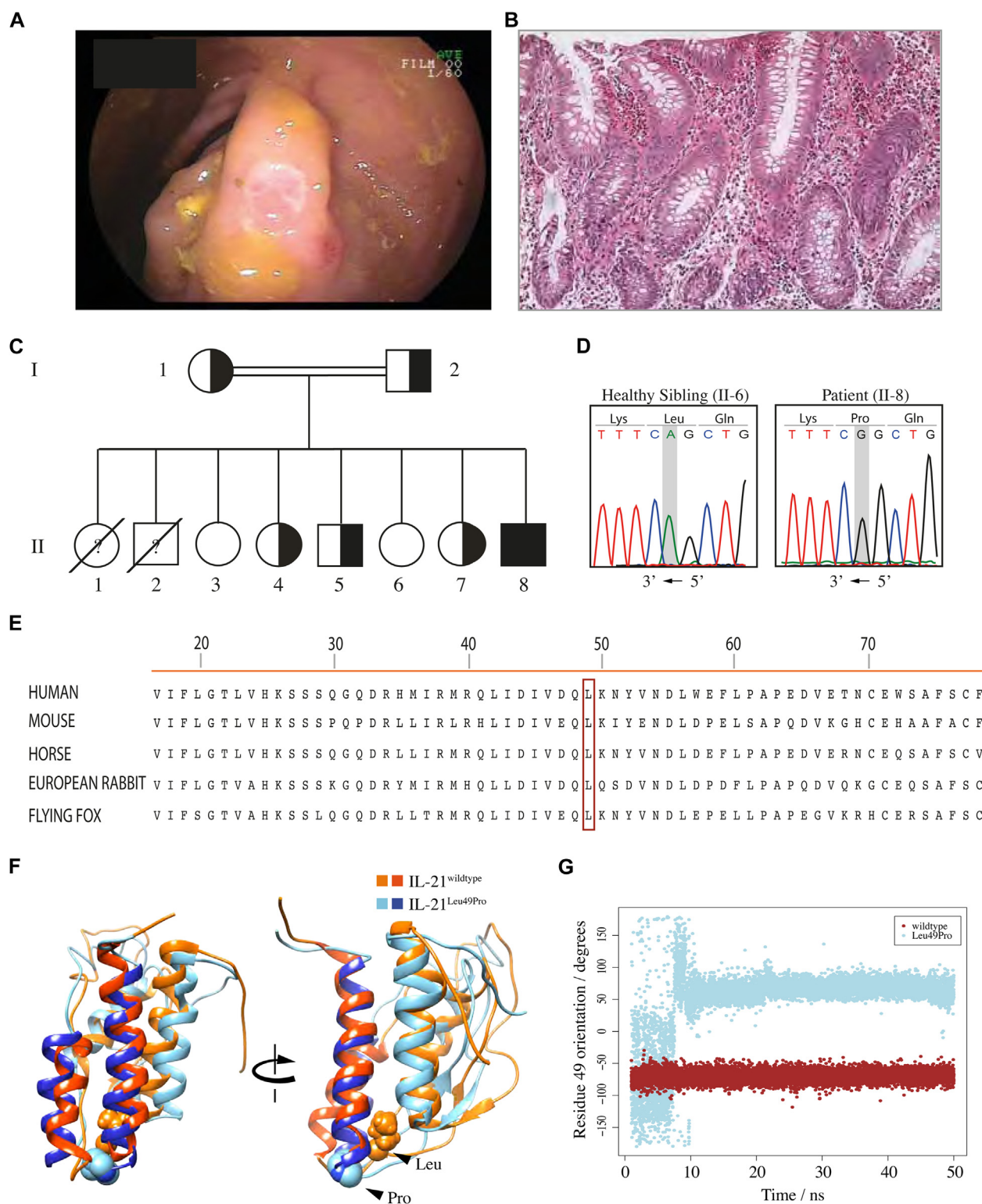


**FIG 1.** Identification of IL-21 deficiency. **A**, Colonoscopy. **B**, Histology consistent with Crohn disease. **C**, Pedigree of index family. *Solid portions of symbols* mark IL-21<sup>Leu49Pro</sup> segregation pattern. **D**, Sanger chromatograms displaying the missense mutation in IL21 in the patient (II-8) and a healthy sibling (II-6). **E**, Amino acid sequence conservation of IL-21<sup>Leu49Pro</sup> among different species. **F**, Structure of mutant IL-21<sup>Leu49Pro</sup> (blue) overlapped with IL-21<sup>WT</sup> (brown). **G**, Orientation of residue 49 in simulations of WT (brown) and IL-21<sup>Leu49Pro</sup> (blue).

**TABLE I.** Laboratory data of the index patient

|                                | 5 y      | 6 y (IVIG) |
|--------------------------------|----------|------------|
| <b>Immunoglobulins</b>         |          |            |
| IgG (mg/dL)                    | 338      | 567        |
| IgM (mg/dL)                    | 96       | 84         |
| IgA (mg/dL)                    | 67       | 61         |
| IgE (kU/mL)                    | 119      |            |
| IgG <sub>1</sub> (mg/dL)       | 230      |            |
| IgG <sub>2</sub> (mg/dL)       | 60       |            |
| IgG <sub>3</sub> (mg/dL)       | 7        |            |
| IgG <sub>4</sub> (mg/dL)       | 6        |            |
| <b>Complement</b>              |          |            |
| CH50 (U/mL)                    | +        | +          |
| C3 (mg/dL)                     | 0.992    |            |
| C4 (mg/dL)                     | 0.674    |            |
| <b>Antibodies</b>              |          |            |
| <i>Cryptosporidium</i> species | Negative |            |
| <i>Giardia lamblia</i>         | Negative |            |
| Rotavirus                      | Negative |            |
| Adenovirus                     | Negative |            |
| Cytomegalovirus                | Negative |            |
| HIV                            | Negative |            |
| Isohemagglutinin anti-B        | 1/2      |            |
| BCG + PPD                      |          | Negative   |
| Anti-HBs                       | Positive | Positive   |
| ANCA                           |          | Negative   |
| ANA                            |          | Negative   |

ANA, Antinuclear antibody; ANCA, antineutrophil cytoplasmic antibody; IVIG, intravenous immunoglobulin; HBs, hepatitis B virus surface antigen.

peripheral blood of the patient compared with control values (Fig 2, C). Analysis of T-cell subsets revealed normal relative proportions of CD3<sup>+</sup>CD4<sup>+</sup> T<sub>H</sub> cell and CD3<sup>+</sup>CD8<sup>+</sup> cytotoxic T-cell subsets (data not shown) but an increase in CD3<sup>+</sup> TCRγδ cell numbers (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The cell receptor Vβ repertoire was found to be polyclonal (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), and cell proliferation induced by various stimuli was found to be normal (Fig 2, D, left panel), except for tetanus toxoid stimulation, despite the fact that the patient has a history of tetanus toxoid vaccinations (Fig 2, D, right panel).

### IL-21<sup>Leu49Pro</sup> does not induce IL-21R signaling, proliferation, and activation of B cells

The effects of Leu49Pro on the activity of the cytokine were analyzed by comparing *in vitro* B- and T-cell responses induced by equal amounts of recombinant mutant (IL-21<sup>Leu49Pro</sup>) or WT (IL-21<sup>WT</sup>) protein produced in HEK-EBNA cells (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). A Jurkat T-cell line was used to test STAT3 phosphorylation to exclude donor-dependent differences in T-cell responses. Stimulation with either commercially available recombinant IL-21 (IL-21<sup>rec</sup>) or IL-21<sup>WT</sup> phosphorylated the STAT3 tyrosine residue 705, whereas IL-21<sup>Leu49Pro</sup> did not induce STAT3 phosphorylation at this position (Fig 3, A). Differences between IL-21<sup>WT</sup> and IL-21<sup>Leu49Pro</sup> in activating B cells were tested on normal donor cord blood-derived B cells.

Proliferation of total CD19<sup>+</sup> (Fig 3, B) and transitional cord blood-derived CD19<sup>+</sup> (Fig 3, C) B cells, as well as proliferation of memory and plasmablast subsets (see Fig E5 in this article's

Online Repository at [www.jacionline.org](http://www.jacionline.org)), were strongly decreased on activation with mutant IL-21<sup>Leu49Pro</sup>.

### IL-21-deficient B cells can be activated and undergo class-switch recombination

PBMCs were stimulated for 3 days with CD40L and IL-21 or alternatively with CD40L and IL-4 and tested for the expression of the activation markers CD69, CD86, and CD95 and class-switch recombination indicated by surface expression of IgG and IgA, respectively, to exclude intrinsic defects affecting the patient's B cells.

As expected, the expression pattern of the activation markers was normal in the B cells of the patient (Fig 4, A). Similar to control B cells, they proliferated and differentiated into IgG<sup>+</sup>/IgA<sup>+</sup> cells in response to IL-21, strongly suggesting that the patient's B cells have an intact capacity to develop into plasma cells and memory B cells in response to IL-21R signaling (Fig 4, B).

## DISCUSSION

Combining homozygosity mapping with exome sequencing, we uncovered IL-21 deficiency as a novel monogenetic cause of severe, early-onset IBD associated with a CVID-like primary immunodeficiency.

IL-21 belongs to the type I cytokine family and acts on many cells of the hematopoietic system.<sup>31</sup> IL-21 is predominantly produced by antigen-activated CD4<sup>+</sup> T cells and activated B-helper neutrophils.<sup>32</sup> B-helper neutrophils secrete IL-21 to promote T cell-independent B-cell responses against microbes.<sup>32</sup> Therefore our finding that IL-21 deficiency causes early-onset IBD suggests that IL-21 plays a critical role in the cooperation between the innate and adaptive immunity in the gut.

IL-21R is expressed by T, B, and natural killer (NK) cells, subsets of myeloid cells, and keratinocytes.<sup>33–36</sup> IL-21 binding to IL-21R induces primarily STAT1 and STAT3 phosphorylation.<sup>30</sup> Therefore it differs from other type I cytokines, such as IL-2, IL-7, IL-9, and IL-15, because these cytokines induce STAT3 and STAT5 activation, whereas IL-4 activates STAT6.<sup>37,38</sup> IL-21 does not seem to influence T<sub>H</sub>1 or T<sub>H</sub>2 polarization in CD4<sup>+</sup> T cells,<sup>39,40</sup> but it appears to be critical for the proliferation and activation of both naive and memory CD8<sup>+</sup> T cells.<sup>40</sup> In NK cells, IL-21 contributes to phenotypic and functional maturation by inducing the expression of killer immunoglobulin-like receptors, perforin expression, and IFN-γ secretion.<sup>41,42</sup> In CD40L-activated B cells, IL-21 strongly promotes proliferation, immunoglobulin class-switch recombination, and immunoglobulin secretion<sup>39,43</sup> while inhibiting the IL-4-driven induction of germline transcripts from the IgE constant region.<sup>44,45</sup> Accordingly, *Il21r*<sup>−/−</sup> knockout mice show dysgammaglobulinemia characterized by low IgG<sub>1</sub> but higher IgE levels.<sup>39</sup> In human subjects IL-21R deficiency has recently been reported to result in primary immunodeficiency characterized by defective B-cell class-switching, aberrant T-cell cytokine production, and NK cell cytotoxicity.<sup>46</sup>

Surprisingly, in contrast to the patients reported with IL-21R deficiency, the IL-21-deficient patient described here had early-onset IBD, which masked primary immunodeficiency found at a later age. In addition, 2 of the patient's siblings died of IBD within the first year of life, underlining the consistency of early-onset IBD presentation in this pedigree. Furthermore, to date, the patient has

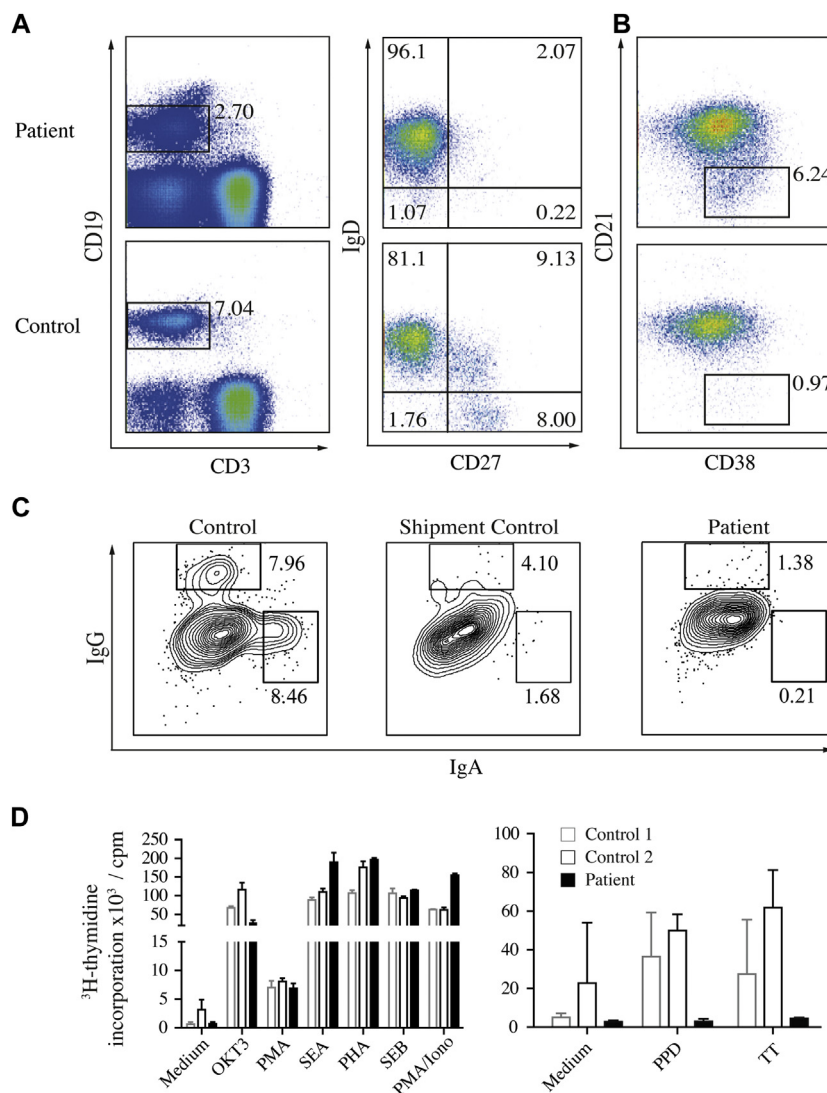


**FIG 2.** Immunologic phenotype of the patient. **A** and **B**, Proportion of total B cells and IgD<sup>+</sup>CD27<sup>+</sup> and IgD<sup>-</sup>CD27<sup>+</sup> memory B cells in peripheral blood from the patient (Fig 2, A, upper panel) and transitional B cells (Fig 2, B) compared with a healthy donor. **C**, Percentage of IgG<sup>+</sup> and IgA<sup>+</sup> class-switched cells in peripheral blood from patients and control subjects. **D**, T-cell proliferation: stimulation for 3 (left panel) and 7 (right panel) days. Iono, ionomycin; PMA, phorbol 12-myristate 13-acetate; PPD, purified protein derivative; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin; TT, tetanus toxoid.

not shown any signs of cholangitis and was negative for *Cryptosporidium* species infection, one of the most striking observations in 3 of 4 of the described IL-21R-deficient patients.<sup>46</sup> However, 2 of the 4 patients with IL-21R deficiency also presented with diarrhea, which was possibly attributed to *Cryptosporidium* species infection and therefore not thought to be a primary cause of defective IL-21R-dependent signaling. *Cryptosporidium* species infections are common in patients with combined immunodeficiencies<sup>47</sup> and may have altered the clinical features of the immunodeficiency in the reported IL-21R-deficient patients. In addition, we cannot exclude underlying differences in the gut microbiota, which are essential nongenetic players in the development of IBD.<sup>48,49</sup> Future studies will need to address the complex interplay of the microbiome on the course of monogenetically determined immunodeficiency disorders.

The primary immunodeficiency caused by mutation in the *IL21* gene was characterized by reduced relative numbers of B cells and dramatically reduced class-switched memory B-cell populations,

increased IgE levels, and hypogammaglobulinemia in peripheral blood. These findings are in line with the observations from murine models<sup>50</sup> and human IL-21 deficiency.<sup>46</sup> Although proliferation of IL-21<sup>Leu49Pro</sup>-expressing T cells appeared normal on stimulation with several common stimuli, stimulation with specific agents, such as tetanus toxoid, illustrated specific defects in T-cell proliferation, thus recapitulating the findings described for IL-21R deficiency.<sup>46</sup>

Interestingly, approximately 20% of patients with CVID have autoimmunity, which often results in colitis and is associated with severely increased morbidity.<sup>14</sup> It has been hypothesized that IL-21 has immunosuppressive activities by inducing IL-10 secretion.<sup>51</sup> Similar to IL-10 or IL-10 receptor deficiency, IBD in the IL-21-deficient patient manifested in the first year of life and was characterized by a severe phenotype.<sup>6,7,12,52</sup> It has been shown previously that synergistic stimulation of bone marrow- or spleen-derived NK cells with IL-21 and IL-2 or IL-15 leads to increased IL-10 secretion, suggesting that the IBD might be

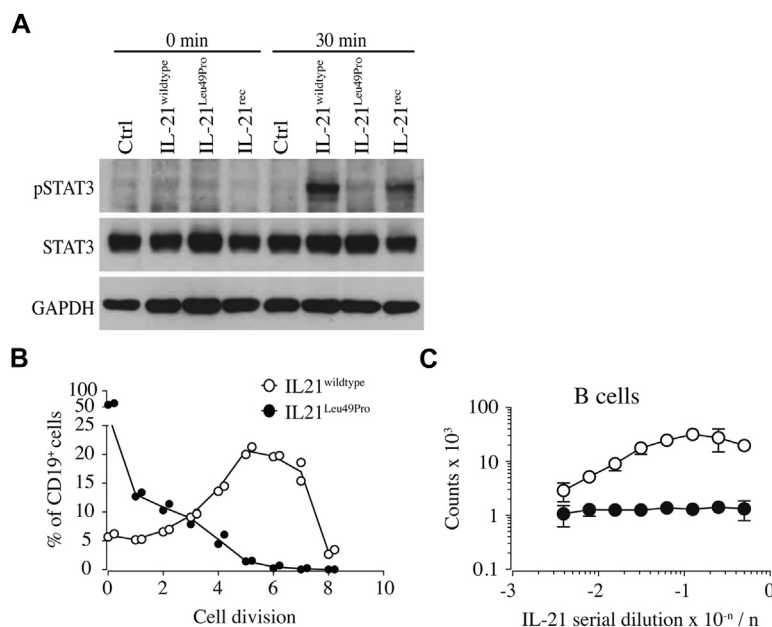


**FIG 3.** Functional consequences of IL-21 deficiency. **A**, Western blot of phospho-STAT3 in Jurkat cells after stimulation with IL-21<sup>WT</sup>, IL-21<sup>Leu49Pro</sup>, and commercially available IL-21 (IL-21<sup>rec</sup>). *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase. **B**, Flow cytometry-based proliferation analysis of carboxyfluorescein succinimidyl ester-labeled cord blood-derived CD19<sup>+</sup> B cells after stimulation with IL-21<sup>WT</sup> (open circles) and IL-21<sup>Leu49Pro</sup> (solid circles). **C**, IL-21-dependent proliferation of cord blood-derived B cells 6 days after stimulation with IL-21<sup>WT</sup> (open circles) or IL-21<sup>Leu49Pro</sup> (solid circles).

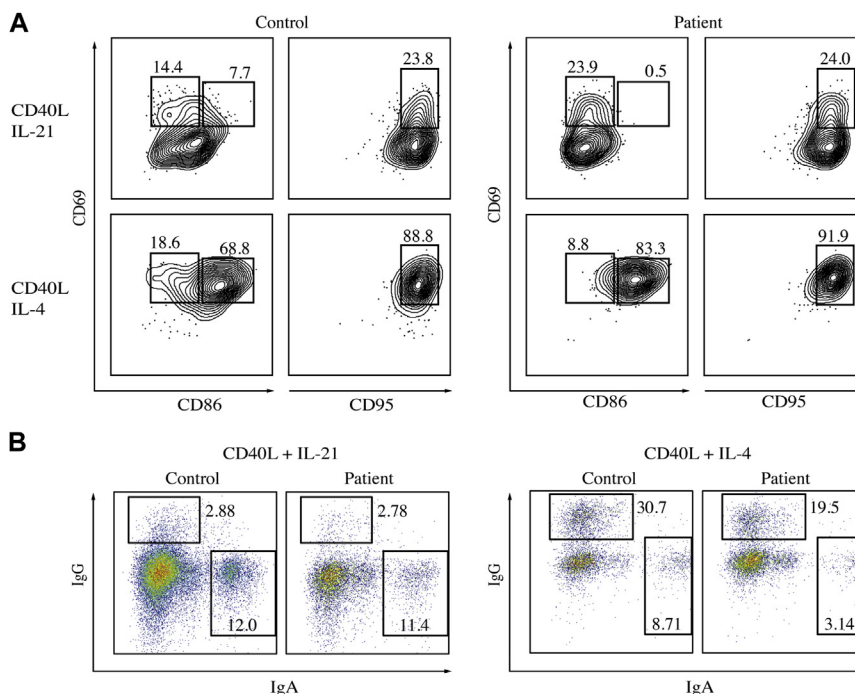


**FIG 4.** Normal activation and class-switch recombination potential of IL-21-deficient B cells. **A**, Flow cytometric analysis of PBMCs from control subjects and patients stimulated with either CD40L and IL-21 (upper panel) or CD40L and IL-4 (lower panel). CD19<sup>+</sup> B cells were stained for the activation markers CD69, CD86, and CD95 after 3 days of stimulation. **B**, Capacity of CD19<sup>+</sup> B cells from control subjects and patients to undergo class-switch recombination into IgG<sup>+</sup> and IgA<sup>+</sup> cells after 6 days of stimulation.

due to decreased IL-10 secretion caused by IL-21 deficiency.<sup>37</sup> Nevertheless, the molecular mechanisms of how IL-21 deficiency leads to gut inflammation remain elusive.

IL-21 has emerged as a critical cytokine regulating multiple arms of the immune system (reviewed by Leonard and Spolski<sup>30</sup>). More recently, interfering with IL-21 signaling has been proposed

as a treatment option for patients with various autoimmune diseases, including systemic lupus erythematosus<sup>53</sup> and rheumatoid diseases.<sup>54,55</sup> However, our observation that lack of functional IL-21 is associated with an immunodeficiency with considerable morbidity raises an important concern for such therapeutic strategies.

In principle, IL-21 deficiency might be amenable to allogeneic hematopoietic stem cell transplantation to correct for the disease<sup>6,52</sup>; however, given the currently stable clinical condition, this has not been performed in the patient to date but remains an option for the future. In contrast to IL-21R deficiency, treatment with recombinant IL-21 might represent an alternative experimental strategy for treatment of IL-21 deficiency in a similar manner, as has been proposed for metastatic cancer, including metastatic melanoma<sup>56</sup> and renal cell carcinoma.<sup>57-59</sup> This might be particularly attractive for scenarios in which the patient's clinical status is incompatible with allogeneic hematopoietic cell transplantation.

In conclusion, here we identify deficiency of IL-21 as a novel cause of primary immunodeficiency and early-onset IBD, thereby further underlining the critical importance of tight control of immune homeostasis for inflammatory processes in the gut. Future studies will show whether targeting the affected pathways is a therapeutic option for IL-21 deficiency and related disorders.

We thank the family for their participation in this study and all clinicians who helped to manage the patient through the course of the disease. We also thank Linda Stöger for exclusion of coding mutations in *IL10*, *IL10RA*, and *IL10RB*, as well as Raphael Ott for assistance with library preparation for exome sequencing and Cecilia Domínguez Conde for critical proofreading of the manuscript. In addition, we would like to thank Frank Zaucke and his team for providing the HEK-EBNA system for recombinant protein production.

**Clinical implications: IL-21 deficiency is a novel cause of early-onset IBD and a CVID-like primary immunodeficiency. The observation that lack of IL-21 is associated with an immunodeficiency raises an important concern for therapeutic strategies interfering with IL-21 in patients with immune-mediated diseases.**

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## METHODS

### DNA isolation

Genomic DNA was isolated from whole blood by using a commercially available kit (Wizard Genomic DNA Purification Kit; Promega, Vienna, Austria), according to the manufacturer's instructions.

### Sequencing of *IL10*, *IL10RA*, and *IL10RB*

Sanger sequencing of *IL10*, *IL10RA*, and *IL10RB* was performed according to standard methods. Primers are listed in Table E1. Primers marked with a footnote were described previously in Glocker et al.<sup>E1</sup>

### Homozygosity mapping

SNP-based homozygosity mapping with Affymetrix 6.0 arrays (Affymetrix, Santa Clara, Calif) was performed on genomic DNA from the index patient to map homozygous intervals, as described previously, according to the Affymetrix Genome-Wide Human SNP Nsp/Sty 6.0 protocol.<sup>E2</sup> In brief, 10  $\mu$ L of 50 ng/ $\mu$ L DNA was digested with restriction enzymes and ligated to adaptors. The adaptor-ligated DNA fragments were amplified with a generic primer recognizing the adaptor sequences. The amplified DNA was fragmented, labeled, and hybridized to the Affymetrix Genome-Wide Human SNP 6.0 Array. Results were analyzed with Affymetrix Genotyping Console software.

Homozygosity Mapper ([www.homozygositymapper.org/](http://www.homozygositymapper.org/)) and PLINK<sup>E3</sup> whole-genome data analysis toolset with a window size of 5000 bp (a minimum of 50 SNPs within this region) were used to detect homozygous regions.

### Exome sequencing

Sample preparation was done with the Illumina TruSeq DNA Sample Preparation Guide and the Illumina TruSeq Exome Enrichment Guide version 3 (Illumina, San Diego, Calif). The genomic DNA (1  $\mu$ g) was sheared to fragments of 200 to 300 bp. Blunt ending, adenylation, and adapter ligation to allow the fragments to hybridize onto the flow cell were carried out. Subsequently, exons of the DNA fragments were enriched, and clusters were generated with the Illumina cBot Cluster Generation System according to the TruSeq PE Cluster Kit version 3 Reagent Preparation Guide. The DNA fragment clusters ran in a multiplexed pool with 5 other samples distributed on 3 lanes of the flow cell.

Data analysis was performed as previously described.<sup>E2</sup> In brief, reads were aligned by using Burrows-Wheeler Aligner to the human genome 19. Insertion/deletion realignment was performed, as was Genome Analysis Toolkit (GATK version 1.4)-based quality score recalibration. For single nucleotide variant (SNV) and deletion/insertion variant calling, Unified Genotyper and GATK Variant quality score recalibration were performed. SNV and deletion/insertion variant lists were uploaded to the SeattleSeq Annotation database (accessed January 2012). Variants present in 1000 Genomes and dbSNP build 135 were excluded, and the lists were filtered for nonsense, missense, and splice-site variants present within the homozygous regions only found in the patient.

### Variant validation

Validation of the variants found on the final list of hits was performed by using capillary sequencing on genomic DNA from the patient. The primers for each position were designed with Primer3 (<http://genepipe.ngc.sinica.edu.tw/primer3/>), excluding 60 bp adjacent to the target position and with the product size ranging between 100 and 500 bp. All primers were purchased from Sigma-Aldrich.

PCR amplification of the detected variants was performed with the Expand High Fidelity PCR System (Roche, Basel, Switzerland). Capillary sequencing was carried out with the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany), and the analysis took place on a 3130xl Genetic Analyzer (Applied Biosystems). Sequencer DNA Software version 4.10.1 (Gene Codes, Ann Arbor, Mich) was used for sequence analysis. Segregation analysis of the validated hits was performed in the parents and healthy siblings.

### T-cell CDR3 V $\beta$ spectratyping

TCR V $\beta$  spectratyping was performed according to the method of Pannetier et al.<sup>E4</sup> The primers used were described previously,<sup>E5</sup> with the exceptions indicated in Table E2.

Sequences were acquired with an ABI 3130xl Sequencer (Applied Biosystems) and analyzed with ABI GeneMapper software, version 4.0.

## RESULTS

### Homozygosity mapping

Homozygous intervals detected in the patient with Homozygosity Mapper (Table E3) and PLINK (Table E4). The interval on chromosome 4 in which *IL21* was detected (gray shading) was found with both methods.

### Exome sequencing

Filtering of exome sequencing data for unknown missense, nonsense, or splice-site variants present within the homozygous regions found in the patient revealed 23 SNVs, of which 10 could be validated by using capillary sequencing. For hits in *LOC100132767* (chromosome 16: 29606607 G  $\rightarrow$  R), *CNTNAP4* (chromosome 16: 76311602 G  $\rightarrow$  T), and *C17orf100* (chromosome 17: 6555546 C  $\rightarrow$  G), no unique sequences could be obtained (all positions refer to hg19). Under the assumption of an autosomal recessive trait with full penetrance, perfect segregation with the disease was found for the variant in *IL21*, which is discussed in detail in the main article, as well as in the gene *ENPEP* encoding aminopeptidase A (BP-1/6C3; chromosome 4: 111431495 C  $\rightarrow$  A). The detected variant in *ENPEP* results in an amino acid change from alanine to glutamine at position 430 of the protein. Although it had previously been hypothesized that *ENPEP* might play a role in early B-cell development,<sup>E6</sup> the corresponding knockout mouse showed normal T- and B-cell numbers, displayed normal T cell-dependent and T cell-independent antibodies, and had normal serum immunoglobulin levels.<sup>E7</sup> Therefore this variant was not further considered to be of pathogenic relevance to the patient's disease.

### Modeling of *IL21*

*In silico* modeling of the variant detected in *IL21* was done by using <http://cupsat.tu-bs.de/jsp/nmrpredict.jsp>. This tool enables the prediction of an amino acid change at a certain position of the protein. Of all possible amino acid changes, the exchange to proline, which was detected in the patient, was predicted to be the most unfavorable (gray shading in Table E3).

To obtain a qualitative hypothesis about the difference in protein structure and dynamics of the IL-21 variants, we performed MD simulations of IL-21<sup>WT</sup> and IL-21<sup>Leu49Pro</sup>. Simulations were performed with the coarse-grained model FREADY<sup>E8</sup> implemented in the MOIL<sup>E9,E10</sup> molecular modeling package. We initiated the MD simulations from the NMR structure of IL-21 (PDB id 2OQP)<sup>E11</sup> and let it run for 50 ns at 300 K. Simulations were performed with different degree of flexibility for the protein, ranging from fully free MD simulations to simulations in which backbone dihedral angles of residues further than a flexibility cutoff from residue 49 were constrained to remain similar to the NMR structure. The flexibility cutoff was varied from 5 to 9 Å. All simulations over a range of flexibility cutoffs, including the fully free simulation, resulted in the same qualitative behavior. Results displayed in Fig 1 consider simulations with a flexibility cutoff of 9 Å. At this cutoff

value, the overall folding of IL-21 is highly preserved throughout both simulations (backbone Root-mean-square deviation [RMSD] of WT simulation, approximately 3 Å; backbone RMSD of mutant stimulation, approximately 4 Å; template-modeling [TM] scores,<sup>E12</sup>  $\leq 0.93$  and 0.9, respectively), and the local conformational transition at the mutation point is clearly observable (Fig 1, F). Protein structures were aligned and visualized with the UCSF Chimera tool.<sup>E13</sup>

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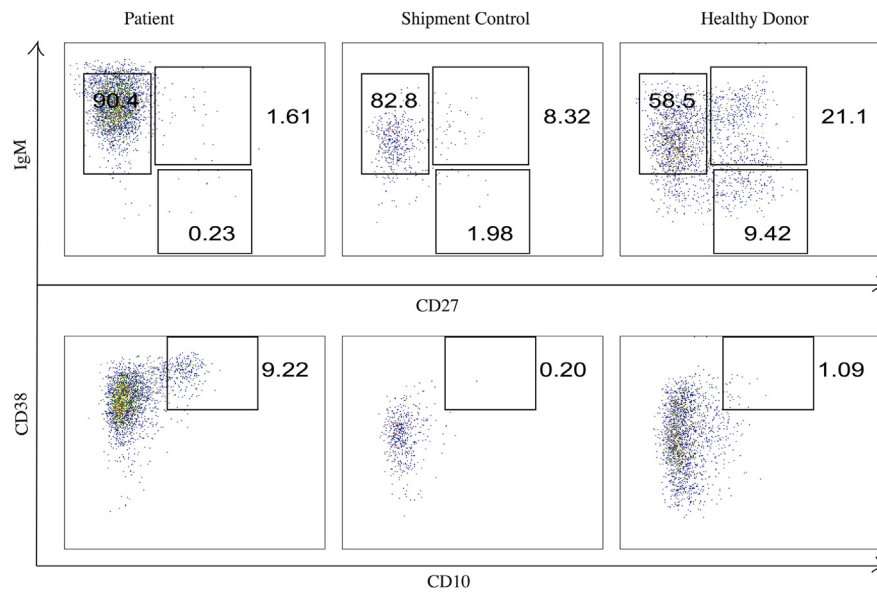


**FIG E1.** Flow cytometric analysis gated on  $CD19^+$  cells displaying decreased  $IgM^+ CD27^+$  non-class-switched memory B cells in the patient compared with a healthy shipment control, as well as a healthy donor (*top*) and increased immature early transitional  $CD38^+ CD10^+$  B cells (*bottom*).

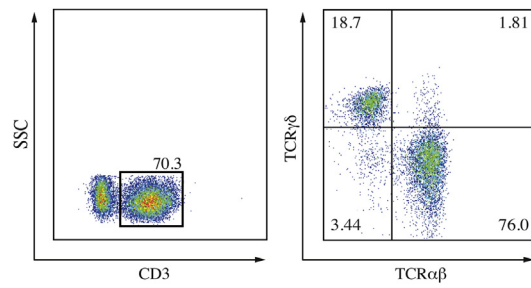


**FIG E2.** Flow cytometric analysis displaying increased proportion of TCR $\gamma\delta^+$ CD3 $^+$  cells. SSC, Side scatter.

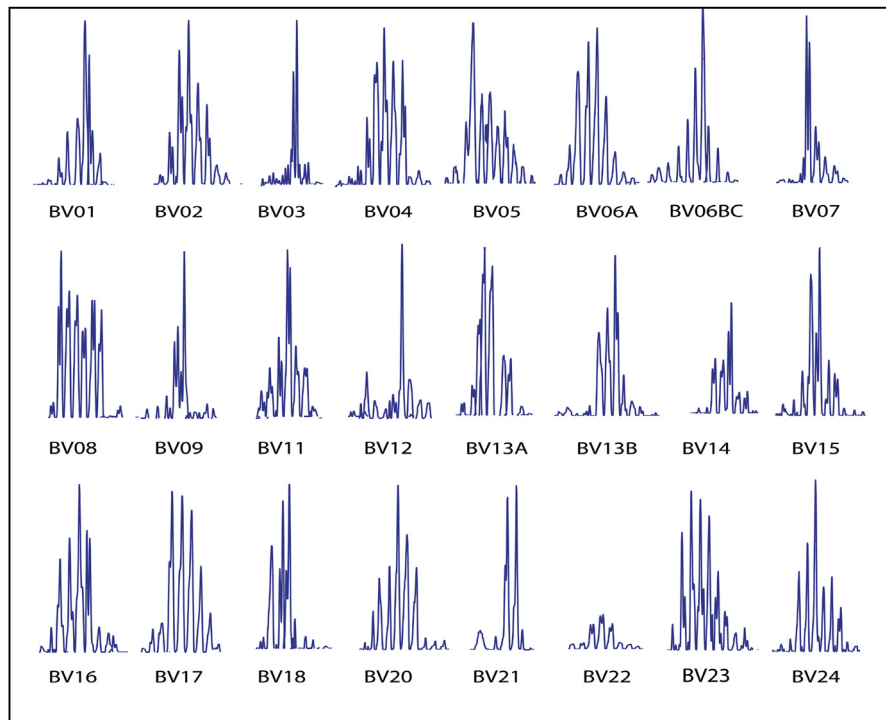


**FIG E3.** TCR V $\beta$  spectratyping analysis. Analysis of the patient shows a normal polyclonal use of the TCR V $\beta$  repertoire.

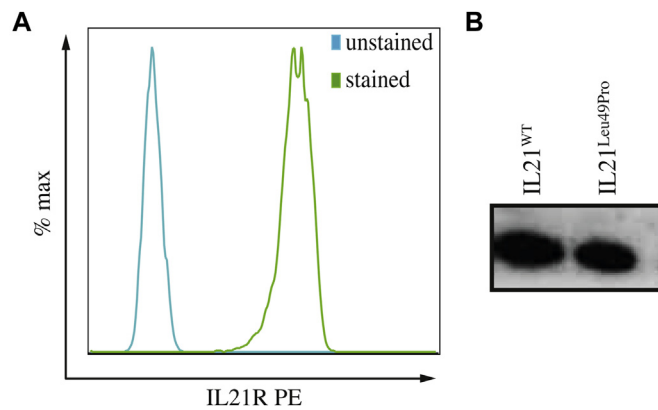


**FIG E4.** IL-21 production by HEK293 cells. **A**, Flow cytometric analysis of IL-21R expression on Jurkat cells. **B**, Western blot analysis of supernatant of IL-21-producing HEK293 cells blotted for the His-tag attached to both with IL-21<sup>Leu49Pro</sup> and IL-21<sup>WT</sup>.

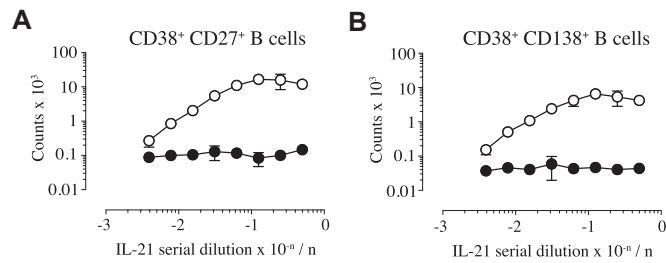


**FIG E5.** Proliferation capacity of the cord blood-derived plasmablast and memory B-cell populations in response to WT (*open circles*) or mutant (*solid circles*) IL-21. Data were gated on CD19<sup>+</sup> cells and analyzed for expression of either CD38 and CD27 (**A**) or CD38 and CD138 (**B**).

**TABLE E1.** Primer sequences

| Gene exon               | Orientation | Sequence (5'-3')         |
|-------------------------|-------------|--------------------------|
| <i>IL10</i> exon 1      | F           | GCCGGGAAACCTTGATTG       |
|                         | R           | GGAATAGGTGTTGGGGATGG     |
| <i>IL10</i> exon 2      | F           | TCCCATCACTGTTGAATCCTC    |
|                         | R           | GACTGAGCCCTTTGTAAACCC    |
| <i>IL10</i> exon 3      | F           | ATCGCTAGAACCAAGCTGTCC    |
|                         | R           | GTGTCTTTGCTGTGTCTGTGG    |
| <i>IL10</i> exon 4      | F           | ACCAGCTTGTCCTTAAGTG      |
|                         | R           | GAATGGGGCCTATTGAGTCC     |
| <i>IL10</i> exon 5.1    | F           | CATGAGCATGAGGGAGGG       |
|                         | R           | TCCGAGACACTGGAAGGTG      |
| <i>IL10</i> exon 5.2    | F           | ATCTTGTCTCTGGGCTTGGG     |
|                         | R           | TGCAGAATTCATTCACCCAC     |
| <i>IL10</i> exon 5.3    | F           | CCTAAATTTGGTTCTAGGCCG    |
|                         | R           | GGCTTCCTTTCTCTGAAATGC    |
| <i>IL10</i> exon 5.4    | F           | TGGATCACTTGAGGTCAGGA     |
|                         | R           | CCTGGGGGTAGGGGGTAG       |
| <i>IL10RA</i> exon 1*   | F*          | GACAGTGGTTCCTCCGTCC*     |
|                         | R*          | CACTGGATGGAGAACTTTAATGG* |
| <i>IL10RA</i> exon 2*   | F*          | GAACCTCCCTTTCTTCTTTGG*   |
|                         | R*          | AGGCAGGTATCTTCCCATGC*    |
| <i>IL10RA</i> exon 3*   | F*          | GGCCTCTTGCGTCTCCC*       |
|                         | R*          | GCAGACATGGTGAGCTATGG*    |
| <i>IL10RA</i> exon 4*   | F*          | ATTCTGGAGGCCAAAGTCTCG*   |
|                         | R*          | AGTTCCCAATGGCACACAAG*    |
| <i>IL10RA</i> exon 5*   | F*          | CTAAAGGCCACCAGCTCTC*     |
|                         | R*          | ACGCGTTTTGGATTGCAC*      |
| <i>IL10RA</i> exon 6*   | F*          | AATGGATTTCATGGGACCAG*    |
|                         | R*          | ACTGGCTGGGAGGAAAAGAG*    |
| <i>IL10RA</i> exon 7.1* | F*          | CGAGCTCTCCTCCTGGG*       |
|                         | R*          | CCTCAGGTAACCCTGGAATG*    |
| <i>IL10RA</i> exon 7.2* | F*          | TGACAGTGGCATTGACTTAGTTC* |
|                         | R*          | GTCCAGGCAGAGGAGCAG*      |
| <i>IL10RA</i> exon 7.3* | F*          | CCTGGGCAGCTTTAACTCAG*    |
|                         | R*          | AGGTTCCCATGTGACCATC*     |
| <i>IL10RA</i> exon 7.4* | F*          | GCTGAAGTCAGCTCAGACCC*    |
|                         | R*          | CAGTGCCCACTGGCTTATC*     |
| <i>IL10RA</i> exon 7.5  | F           | TGGTCATAACTCAGCCCTTG     |
|                         | R           | GTCATGGCTGGATTCCCTG      |
| <i>IL10RA</i> exon 7.6  | F           | AACAAAGGCAGTTCAGTCCAC    |
|                         | R           | AACCGAGTCCTCCATGAGC      |
| <i>IL10RB</i> exon 1*   | F*          | AGGGTAAAGAAGACCCTCAAA*   |
|                         | R*          | CCTAGTTGCGTCTCAGCAG*     |
| <i>IL10RB</i> exon 2*   | F*          | AGCCATAGAGGAGAACCAAGT*   |
|                         | R*          | ACCTAGAGATGACAGCAGTGG*   |
| <i>IL10RB</i> exon 3    | F           | TTAACACAGTTTCCACTCCCG    |
|                         | R           | AAGGCCATCCATTGTGG        |
| <i>IL10RB</i> exon 4    | F           | TCCGTGGACTAATTGTTCTGC    |
|                         | R           | AGTCCATAAGGTGCTGCCAC     |
| <i>IL10RB</i> exon 5    | F           | CCCTGAACTGAGAGGAGCAC     |
|                         | R           | TGAATGAGCTGCCTCAGAAG     |
| <i>IL10RB</i> exon 6*   | F*          | GGATTGTGATGGTTAAAATGC*   |
|                         | R*          | CCCTTTTACAAATAGCCTTCC*   |
| <i>IL10RB</i> exon 7.1* | F*          | ATAGATTTTCCAGCCAGGAGT*   |
|                         | R*          | GCCCTGTTTCTCACAATTAAA*   |
| <i>IL10RB</i> exon 7.2  | F           | CACATCTAGAACTCCAGACCC    |
|                         | R           | TCACTTTGTCACCCAGGC       |
| <i>IL10RB</i> exon 7.3  | F           | GATGGCGCATGCCTATAATC     |
|                         | R           | TGGACATCAAGATGGCAAAC     |

F, Forward; R, reverse.

\*Primers according to Glocker et al.<sup>E1</sup>

**TABLE E2.** Newly designed primers for TCR V $\beta$  spectratyping

| Primer name                                | Sequences                |
|--------------------------------------------|--------------------------|
| Primers for variable regions               |                          |
| BV02                                       | ACATACGAGCAAGGCGTCGA     |
| BV04                                       | CATCAGCCGCCCAAACCTAA     |
| BV07                                       | CAAGTCGCTTCTCACCTGAATGC  |
| BV17                                       | TGTGACATCGGCCCAAAGAA     |
| BV21                                       | GGAGTAGACTCCACTCTAAG     |
| BV24                                       | CCCAGTTTGGAAGCCAGTGACCC  |
| Primers for constant regions               |                          |
| C $\beta$ B1 (used for BV05, BV06BC, BV20) | CGGGCTGCTCCTTGAGGGGCTGCG |
| FAM-marked constant primer                 | ACACAGCGACCTCGGGTGGG     |

**TABLE E3.** Homozygous intervals detected in the index patient (II-8) using Homozygosity Mapper software

| Chromosome | Start      | End        | SNP start  | SNP end    |
|------------|------------|------------|------------|------------|
| 4          | 83395044   | 88826972   | rs7664077  | rs2009152  |
| 4          | 90025980   | 96699508   | rs7691983  | rs10014317 |
| 4          | 96719160   | 109475974  | rs11930626 | rs6835794  |
| 4          | 109481858  | 118949490  | rs7666063  | rs2389493  |
| 4*         | 118949878* | 127531669* | rs2254215* | rs313142*  |
| 4          | 127534757  | 146750966  | rs313139   | rs13149290 |
| 5          | 2757959    | 4885009    | rs1391124  | rs3913374  |
| 5          | 163400151  | 167294665  | rs11135295 | rs13175544 |
| 5          | 167303317  | 169376419  | rs1862198  | rs259917   |
| 5          | 169516018  | 173192744  | rs10073750 | rs791346   |
| 16         | 27166296   | 54177001   | rs16976570 | rs2689265  |
| 16         | 70928314   | 75081551   | rs1774433  | rs4243109  |
| 16         | 76351875   | 78526427   | rs11864692 | rs8055841  |
| 16         | 78527004   | 80747410   | rs2738716  | rs3100179  |
| 16         | 80754387   | 84729485   | rs4580175  | rs7193019  |
| 17         | 410451     | 10054798   | rs9748016  | rs1024370  |
| 17         | 11245099   | 13841519   | rs16944600 | rs4792419  |

\*Interval which contains IL21.

**TABLE E4.** Homozygous intervals detected in the index patient (II-8) by using PLINK

| Chromosome | Start      | End        | SNP start        | SNP end        |
|------------|------------|------------|------------------|----------------|
| 1          | 49222528   | 50674403   | SNP_A-2011849    | SNP_A-8467861  |
| 2          | 72347041   | 74123827   | SNP_A-2313026    | SNP_A-8687775  |
| 2          | 184851796  | 185944619  | SNP_A-2237199    | SNP_A-8537410  |
| 2          | 189306009  | 190951583  | SNP_A-2053595    | SNP_A-8472550  |
| 3          | 41234516   | 42417982   | AFFX-SNP_5800238 | SNP_A-2263719  |
| 3          | 96053560   | 97183817   | SNP_A-1884420    | SNP_A-8495492  |
| 4          | 1052009    | 2647131    | SNP_A-8420001    | SNP_A-8512601  |
| 4          | 2674271    | 4333327    | SNP_A-2168327    | SNP_A-8684751  |
| 4          | 33409962   | 34724345   | SNP_A-8629522    | SNP_A-1976751  |
| 4          | 83403068   | 88824216   | SNP_A-8389116    | SNP_A-1977976  |
| 4          | 88859131   | 90003726   | SNP_A-8588059    | SNP_A-8458421  |
| 4          | 90034030   | 98611720   | SNP_A-8621918    | SNP_A-8561152  |
| 4          | 98665087   | 109472301  | SNP_A-8680070    | SNP_A-8687642  |
| 4          | 109483856  | 113784482  | SNP_A-1847528    | SNP_A-8371169  |
| 4          | 113811255  | 116726594  | SNP_A-8386473    | SNP_A-2252669  |
| 4*         | 116729292* | 127525092* | SNP_A-8648720*   | SNP_A-8445435* |
| 4          | 127539506  | 129888651  | SNP_A-8662340    | SNP_A-8630365  |
| 4          | 129965026  | 142021835  | SNP_A-4272521    | SNP_A-8662873  |
| 4          | 142066636  | 146764719  | SNP_A-1979175    | SNP_A-1932018  |
| 5          | 36344      | 2738455    | SNP_A-8392711    | SNP_A-4273394  |
| 5          | 3642021    | 4819918    | SNP_A-8664849    | SNP_A-1897861  |
| 5          | 44719866   | 45888504   | SNP_A-8604529    | SNP_A-4282307  |
| 5          | 56755502   | 58101645   | SNP_A-1854819    | SNP_A-8396567  |
| 5          | 97874728   | 99039123   | SNP_A-8607625    | SNP_A-2274520  |
| 5          | 163392787  | 167296428  | SNP_A-8708191    | SNP_A-2272719  |
| 5          | 167309980  | 169374909  | SNP_A-1904490    | SNP_A-2197407  |
| 5          | 170274844  | 172957916  | SNP_A-2260629    | SNP_A-1895904  |
| 6          | 26091336   | 27189517   | SNP_A-4273092    | SNP_A-2258495  |
| 7          | 62550165   | 63692343   | SNP_A-8346354    | SNP_A-8330375  |
| 7          | 123016059  | 124061924  | SNP_A-4266575    | SNP_A-8520938  |
| 9          | 133988447  | 137109245  | SNP_A-8379710    | SNP_A-1908201  |
| 9          | 138059244  | 139666473  | SNP_A-8496411    | SNP_A-8690526  |
| 9          | 139839904  | 141071475  | SNP_A-8477786    | SNP_A-8302801  |
| 10         | 22320193   | 23336057   | SNP_A-8553730    | SNP_A-8349587  |
| 10         | 68522925   | 69871319   | SNP_A-2304575    | SNP_A-4275777  |
| 12         | 37857751   | 38957140   | SNP_A-2298756    | SNP_A-1914010  |
| 12         | 88317831   | 89373308   | SNP_A-4268475    | SNP_A-8471938  |
| 14         | 24239341   | 26640934   | SNP_A-2037635    | SNP_A-1807016  |
| 14         | 27450705   | 28791689   | SNP_A-8623427    | SNP_A-8319810  |
| 14         | 90646020   | 92838444   | SNP_A-8646350    | SNP_A-2246935  |
| 15         | 42335477   | 43395847   | SNP_A-2004120    | SNP_A-2130382  |
| 15         | 72094856   | 73124491   | SNP_A-8685433    | SNP_A-2276311  |
| 16         | 27842014   | 35205717   | SNP_A-8582579    | SNP_A-8595671  |
| 16         | 46534977   | 54175313   | SNP_A-8350504    | SNP_A-8413639  |
| 16         | 54355601   | 55425383   | SNP_A-8319132    | SNP_A-8390684  |
| 16         | 55442987   | 57154547   | SNP_A-8424883    | SNP_A-2279806  |
| 16         | 67040335   | 68326200   | SNP_A-1812292    | SNP_A-1806333  |
| 16         | 69534688   | 70836370   | SNP_A-4244436    | SNP_A-2004827  |
| 16         | 71103393   | 75046184   | SNP_A-8610073    | SNP_A-8364418  |
| 16         | 76355255   | 78517027   | SNP_A-8560561    | SNP_A-1806599  |
| 16         | 78527065   | 82919690   | SNP_A-4192918    | SNP_A-2027051  |
| 16         | 83379737   | 84730830   | SNP_A-8401789    | SNP_A-8280151  |
| 17         | 6689       | 3205886    | SNP_A-8398136    | SNP_A-2284710  |
| 17         | 3210368    | 5000130    | SNP_A-4196849    | SNP_A-8299342  |
| 17         | 5133734    | 11236566   | SNP_A-8287174    | SNP_A-2224114  |
| 17         | 11259037   | 13837051   | SNP_A-4202679    | SNP_A-1945664  |
| 17         | 19442146   | 20554628   | SNP_A-8344319    | SNP_A-8546621  |
| 17         | 28687196   | 29796197   | SNP_A-1816266    | SNP_A-8616647  |
| 20         | 32773809   | 33831187   | SNP_A-1941912    | SNP_A-8383932  |

\*Interval which contains IL21.

**TABLE E5.** Prediction and effect of an amino acid change in IL-21 at position Leu49

| Amino acid | Overall stability | Torsion     | Predicted $\Delta\Delta G$ (kcal/mol) |
|------------|-------------------|-------------|---------------------------------------|
| GLY        | Destabilizing     | Unfavorable | −2.51                                 |
| ALA        | Destabilizing     | Unfavorable | −1.92                                 |
| VAL        | Destabilizing     | Favorable   | −1.01                                 |
| ILE        | Destabilizing     | Favorable   | −1                                    |
| MET        | Destabilizing     | Unfavorable | −1.56                                 |
| PRO        | Destabilizing     | Unfavorable | −3.92                                 |
| TRP        | Destabilizing     | Unfavorable | −1.53                                 |
| SER        | Destabilizing     | Unfavorable | −1.78                                 |
| THR        | Destabilizing     | Favorable   | −1.41                                 |
| PHE        | Destabilizing     | Favorable   | −1.43                                 |
| GLN        | Destabilizing     | Unfavorable | −1.64                                 |
| LYS        | Destabilizing     | Unfavorable | −1.65                                 |
| TYR        | Destabilizing     | Favorable   | −1.44                                 |
| ASN        | Destabilizing     | Unfavorable | −1.69                                 |
| CYS        | Destabilizing     | Favorable   | −1.44                                 |
| GLU        | Destabilizing     | Unfavorable | −1.72                                 |
| ASP        | Destabilizing     | Unfavorable | −1.8                                  |
| ARG        | Destabilizing     | Unfavorable | −1.6                                  |
| HIS        | Destabilizing     | Unfavorable | −1.51                                 |