

Mechanistic Associations of a Mild Phenotype of Immunodysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome

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Background & Aims: The syndrome of immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is a rare disorder resulting in the expression of multiple autoimmune and allergic features. Early onset enteropathy and type 1 diabetes (T1D) are the most common clinical features. The IPEX syndrome is caused by mutations of the FOXP3 gene, which is essential for the development of regulatory T cells (Treg). We describe 2 unrelated patients with IPEX syndrome with a mild clinical phenotype and with novel FOXP3 mutations and the phenotypic and functional characterization of their Treg cells. **Methods:** The FOXP3 gene was analyzed by sequencing amplicons from genomic DNA. Treg cells were characterized by evaluating the number of CD4⁺CD25⁺ T cells and their functional ability to suppress the proliferation of autologous CD4⁺CD25[–] effector T cells stimulated with anti-CD3 and anti-CD28 antibodies. **Results:** A 7-year-old boy and a 24-year-old man presented with autoimmune enteropathy characterized by early onset persistent diarrhea not associated with T1D or other endocrinopathies. These 2 patients carry novel FOXP3 mutations that do not abrogate the function of the forkhead domain. They have normal numbers of CD4⁺CD25⁺ T lymphocytes, however, these show severely defective suppressive function in vitro. **Conclusions:** Our 2 patients show that IPEX patients may present with early onset enteropathy and long-term survival without T1D or other endocrinopathies. This milder phenotype may be associated with FOXP3 mutations that do not abrogate the function of the forkhead domain.

The syndrome of immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is characterized by multiple autoimmune and allergic features.^{1–3} Early onset enteropathy, with villous atrophy and infiltrating inflammatory cells, is the most common manifestation. It is difficult to treat and is the primary cause

of death. Early onset type 1 diabetes (T1D), with autoantibodies to islet antigens, usually is present and other autoimmune endocrinopathies are common, together with thrombocytopenia, hemolytic anemia, and arthritis.^{1,4} Asthma and eczema, with increased serum immunoglobulin (Ig)E levels, are frequent. Most reported patients with IPEX have died in infancy or early childhood.

IPEX is caused by mutations of the FOXP3 gene encoding a transcription factor, named *scurfin*, of the forkhead family.⁵ Scurfin is a DNA-binding protein that suppresses cytokine gene expression in activated T lymphocytes, possibly through direct repression of NFAT.⁶ In human beings, 13 FOXP3 mutations have been reported.⁴ Ten disrupt the forkhead domain, 2 affect the leucine zipper domain, and 1 affects the first polyadenylation signal. The forkhead domain is required, both in humans and mice, for FOXP3 function.⁶ The impact of mutations in the leucine zipper domain and in the polyadenylation site on expression and function of FOXP3 still is unknown.

FOXP3-deficient mice have a disease similar to IPEX.⁷ In mice, FOXP3 is essential for the development of CD4⁺CD25⁺ regulatory T cells (Treg) and transplantation of Tregs from a normal strain rescues the clinical phenotype.⁸ Human Treg cells, also included in the CD4⁺CD25⁺ population, inhibit proliferation and cytokine production of effector T cells and have been implicated in several immune-mediated disorders.⁹ We report

Abbreviations used in this paper: ANA, antinuclear antibody; bp, base pair; cDNA, complementary DNA; ESR, erythrocyte sedimentation rate; Ig, immunoglobulin; IPEX, immunodysregulation, polyendocrinopathy, enteropathy, X-linked; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; T1D, type 1 diabetes; Treg, regulatory T cells.

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2 unrelated patients with IPEX and a mild clinical phenotype characterized by early onset enteropathy, absence of T1D or other endocrinopathies, and prolonged survival. These patients carry novel FOXP3 mutations that do not disrupt the forkhead domain and show a functional defect of CD4⁺CD25⁺ Treg cells.

Case Reports

Patient 1

A 22-year-old man was referred because of recurrent diffuse joint pain associated with the presence of antinuclear antibodies (ANAs). The mother died at 42 years of age of intestinal lymphoma. The patient had 3 brothers. One brother died at 1 month of age from intractable diarrhea; diabetes was not reported. The other 2 brothers, both asymptomatic, had biopsy examination–proven celiac disease and responded to a gluten-free diet. One brother died at 14 years of age in an accident; the other brother, aged 26 years, was lost to follow-up evaluation.

At 18 days of age, patient 1 developed diarrhea and severe malnutrition. Parenteral nutrition and a subsequent diet with a peptide formula reportedly were associated with improvement and he was maintained on a gluten-free diet. At 5 years, after reintroduction of a full gluten-containing diet, abdominal pain, diarrhea, and failure to thrive relapsed. An intestinal biopsy examination showed subtotal villous atrophy and mononuclear cell infiltrate in the lamina propria (Figure 1A). Immunohistochemistry subsequently was performed and revealed an intraepithelial CD3⁺CD8⁺ cell count of 42/100 epithelial cells (upper limit of normal, 40) (Figure 1B). High titers of antigliadin IgG and antiendomysial antibodies were found. Celiac disease was diagnosed. After an initial clinical improvement with a gluten-free diet, diarrhea rapidly relapsed and failure to thrive persisted. Two years later a second intestinal biopsy examination showed similar findings (not shown). IgE levels were high (200 mU/mL; normal values for age, <60 mU/mL), reportedly in the absence of allergy. Diarrhea and failure to thrive persisted. At 15 years, the patient developed arthralgia and transient arthritis. Antienterocyte antibodies were detected for the first time in this patient (immunofluorescence on human duodenum; Dr. E. Bosi, San Raffaele Research Institute, Milan, Italy). A diagnosis of autoimmune enteropathy was made and treatment with glucocorticoids and azathioprine was started with partial improvement. At 18 years the patient developed well-defined, scaly, erythematous plaques on the trunk and limbs. A skin biopsy examination showed parakeratosis, Poitrier-like epidermal

microabscesses, and mononuclear cell infiltrate in perivascular aggregates, consistent with psoriasiform dermatitis (Figure 1E).

Chronic enteropathy remained active until admission to our unit despite continuous treatment with glucocorticoids, immunosuppressants (cyclosporin A, tacrolimus), and infliximab, alone or in combination, and intermittent parenteral nutrition. At admission, the patient was receiving prednisone, cyclosporin A, and nightly parenteral nutrition. His height was 164 cm (25th–50th percentile), and his weight was 48 kg (10th–25th percentile). He was in fair general condition, presenting with weakness and mild signs of dehydration. Maculopapular lesions were present over the limbs and trunk. The liver was mildly enlarged. The white blood cell count was 10,570/mm³, hemoglobin level was 12.1 g/dL, platelet count was 219,000/mm³, erythrocyte sedimentation rate (ESR) was 28 mm/h (normal, <20 mm/h), and the C-reactive protein level was 39 mg/L (normal, <5 mg/L). Metabolic acidosis (pH 7.30; bicarbonate, 13.3 mEq/L) and renal failure (serum creatinine level, 2.59 mg/dL; urea nitrogen level, 42 mg/dL) were present. Blood electrolyte, glucose, and liver enzyme levels were normal. ANAs were positive (1:160; homogeneous pattern). Antitransglutaminase antibodies were negative. The patient was treated with 3 intravenous methylprednisolone pulses (1 g/day) and parenteral rehydration. He was discharged 1 week later in good general condition with prednisone (1 mg/kg/day in 1 daily dose), tacrolimus (.04 mg/kg/day), azathioprine (2 mg/kg/day), and nightly parenteral nutrition. His weight was 50 kg, creatinine level was 1.45 mg/dL, and bicarbonate level was 22.0 mEq/L.

Patient 2

A 5-year-old boy was referred to our unit because of pain in the wrist and the left proximal interphalangeal joint of the second finger and diffuse arthralgia. His 12-year-old brother was healthy. Severe chronic diarrhea had appeared at 14 months of age. An intestinal biopsy examination showed dysmorphic villi with no atrophy and a marked mononuclear cell infiltrate in the lamina propria (Figure 1C) and an intraepithelial CD3⁺CD8⁺ cell count of 60/100 epithelial cells (upper limit of normal, 40) (Figure 1D). Antigliadin and antiendomysial antibodies were negative. The patient did not improve on a gluten-free diet. IgE levels were 74 mU/mL (normal value for age, <10 mU/mL). Antienterocyte antibodies were negative. ANAs were present (1:5120, homogenous pattern). The patient was diagnosed with an unspecified autoimmune enteropathy and treatment with prednisone, azathioprine, and exclusive intravenous nutrition

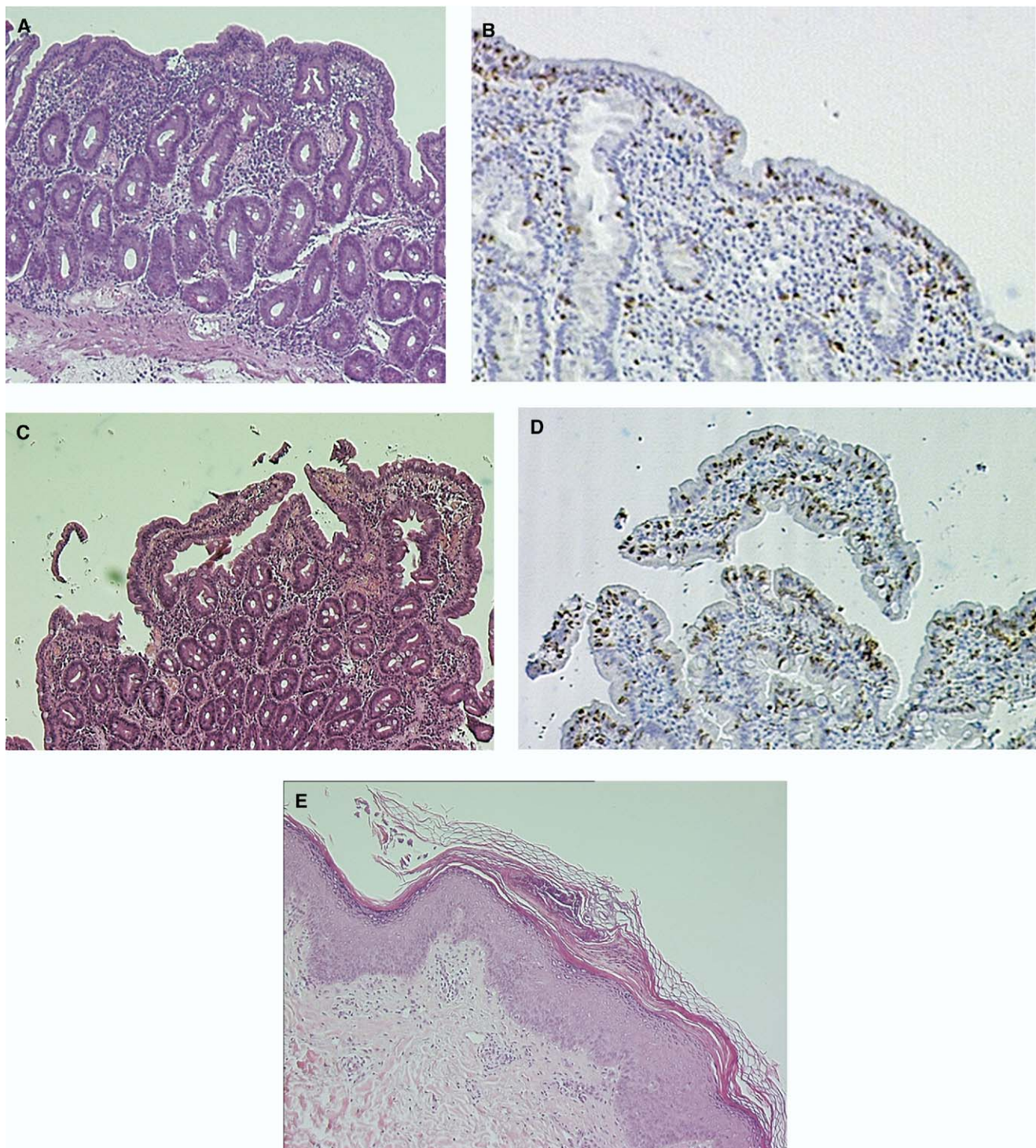


Figure 1. Intestinal biopsy specimen of (A, B) patient 1 and (C, D) patient 2, and a skin biopsy specimen of (E) patient 1. (B, D) Immunohistochemistry of the intestinal biopsy specimen with an anti-CD3 antibody.

was started. Substantial clinical improvement and weight gain ensued. At 18 months of age the patient developed pneumonia with pleuritis, and pericarditis a month later. He was treated with prednisone and azathioprine; treatment was tapered and eventually with-

drawn at 3 years and 5 months. Since then he had been growing satisfactorily on a full gluten-containing diet.

At admission, he was in good clinical condition. His height was 110 cm (50th–75th percentile) and his weight was 16 kg (10th–25th percentile). Physical ex-

Table 1. CD4⁺CD25⁺ T Cells in Patients With IPEX Syndrome, Healthy Controls, and in Patients With Autoimmune Diseases Treated With Glucocorticoids and Immunosuppressants

	CD25 ⁺ total (% of CD4 ⁺)	CD25 ⁺ bright (% of CD4 ⁺)	CD69 ⁺ (% of CD4 ⁺ CD25 ⁺)
Healthy controls (range)	6.5–10.7	.48–1.72	.6–4.2
IPEX (patient 1)	7.3	.91	1.95
IPEX (patient 2)	6.6	1.01	1.79
Autoimmune/treatment			
Control 1	8.53	.62	2.41
Control 2	9.2	1.99	1.89
Control 3	6.92	1.05	1.29
Control 4	10.38	2.40	.51

Autoimmune/treatment: control 1, systemic juvenile idiopathic arthritis treated with prednisone (.4 mg/kg/day) and cyclosporin A (4 mg/kg/day); control 2, juvenile dermatomyositis treated with cyclosporin A (5 mg/kg/day); control 3, systemic juvenile idiopathic arthritis treated with prednisone (.1 mg/kg/day) and cyclosporin A (5 mg/kg/day); control 4, systemic lupus erythematosus treated with prednisone (.4 mg/kg/day) and azathioprine (2 mg/kg/day).

amination showed moderate swelling of the wrists and of the left proximal interphalangeal joint of the second finger. No other signs or symptoms were present. Blood cell counts and liver and kidney functions were normal. ESR was 34 mm/h, the C-reactive protein level was 3.3 mg/L. ANAs were confirmed at a titer of 1:5120 with a homogenous pattern. He was treated with naproxen (15 mg/kg/day). In the subsequent weeks, arthritis and arthralgia persisted and he presented with occasionally watery diarrhea. Treatment with prednisone and azathioprine was started and was followed by a rapid resolution of arthritis and diarrhea. After 2 years, the patient is presently a 7-year-old boy in good health, regularly attending school, who has not presented with recurrences of his enteropathy, and is being treated with azathioprine (2 mg/kg/day) and prednisone (presently .2 mg/kg/day on an alternate-day regimen). ANAs still are present (1:2560). The IgE level is 7.6 IU/mL (normal values for age, <100 IU/mL).

Additional Information

The following investigations were normal or negative in both patients: oral glucose tolerance test, hemoglobin-A1c, fructosamine, insulin-like growth factor, dehydroepiandrosterone-S, thyroid functions, including a thyroid ultrasound, antibodies to glutamic acid decarboxylase, thyroid (thyroglobulin and thyroid-peroxidase), smooth muscle, liver-kidney microsomes, mitochondria, extractable nuclear antigens, cardiolipin, and neutrophil cytoplasm.

Materials and Methods

Mutation Analysis

The 11 exons, including the intron-exon junctions, of the FOXP3 gene were amplified from genomic DNA by polymerase chain reaction (PCR), sequenced by the Big Dye

Terminator kit version 1.1 (Applied Biosystems, Warrington, Cheshire, UK) and the sequences were run on an ABI Prism 377 (Applied Biosystems). A panel of 200 control men was analyzed by denaturing high pressure liquid chromatography to exclude that the nucleotide variations detected in the 2 patients were polymorphisms.

For the expression analysis in patient 1, the total RNA was obtained from peripheral blood mononuclear cells (PBMCs) using TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription was performed with the Advantage reverse transcription for PCR kit (Clontech, Palo Alto, CA). Complementary DNA (cDNA) spanning from exons 1 to 5 was amplified by PCR, with specific primers. Transcripts were cloned by TOPO TA Cloning kit (Invitrogen, Life Technologies Inc.) and selected colonies, representing all the PCR products observed, were sequenced.

Evaluation of the Phenotype and Function of Regulatory T Cells

At the time of sampling, patient 1 was receiving prednisone (.5 mg/kg/day) and cyclosporin A (7 mg/kg/day), and patient 2 was receiving prednisone (.2 mg/kg/day) and azathioprine (2 mg/kg/day). Two independent blood samples from the 2 patients were analyzed. Samples from 4 patients with autoimmune diseases treated with the same immunosuppressants (Table 1) were used as disease/treatment controls. Ten healthy young adults (age, 22–40 y) served as normal controls.

PBMCs were prepared by centrifugation on Ficoll-Hypopaque (Sigma Chemicals, St. Louis, MO). Monoclonal antibodies for flow cytometry and sorting were from Becton-Dickinson (San Jose, CA). For flow cytometry, 5×10^5 PBMCs were stained with appropriate antibody combinations of fluorescein, phycoerythrin, allophycocyanin, and cytochrome-conjugated monoclonal antibodies; in particular, FN50 (anti-CD69), M-A251 (anti-CD25), RPA-T4 (anti-CD4), UCHT1 (anti-CD3), G42-8 (anti-CD8), HIB19 (anti-CD19), and 3G8 (anti-CD16). Cells were analyzed by FACSCalibur (Becton-Dickinson). For sorting, PBMCs were stained with fluorescein

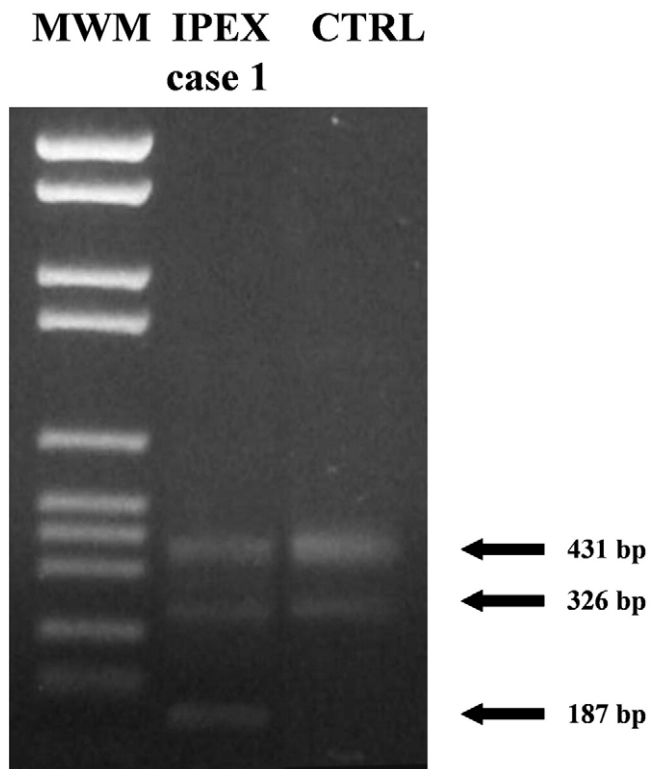


Figure 2. FOXP3 transcripts in patient 1 and in 1 healthy control. The 431-bp fragment corresponds to the full-length cDNA produced by healthy patients; the 326-bp transcript found in the patient and in some of the normal controls represents an alternative splicing with skipping of exon 2 and conserved frame. The 187-bp transcript skips exons 2 and 3 and results in an altered reading frame with a premature stop.

isothiocyanate-conjugated anti-CD8, CD16, and CD19 antibodies, and phycoerythrin-conjugated anti-CD25 antibodies. CD4⁺ T cells were identified as CD8, CD16, CD19, and CD25 negative. The CD4⁺CD25⁺ T cells, containing Treg cells, were negative for all markers, except for CD25. The cell populations were separated using the FACSVantage SE (Becton-Dickinson). Sorted T cells (CD4⁺ or Treg-depleted CD4⁺) were cultured as described previously¹⁰ for 4 days in triplicate in 96-well plates in the presence of irradiated (3000 rads) autologous PBMCs in serum-free X-VIVO-15 medium (Bio-Whittaker Inc, Walkersville, MD) and stimulated with 2 μ g/mL of soluble anti-CD3 and .15 μ g/mL of soluble anti-CD28 (Becton-Dickinson). During the final 16 hours, .5 μ Ci/well of ³H-thymidine was added and proliferation was evaluated by thymidine uptake.

Results

Mutation Analysis

Patient 1 carries an A→G transition at +4 in intron 3 (454+4A>G), very close to the splice site. Reverse-transcription PCR was performed on RNA from PBMCs to obtain cDNA corresponding to exons 1–5.

Three cDNA transcripts of 431, 326, and 187 base pairs (bp) were detected (Figure 2). Cloning and sequencing of the 3 fragments showed that the 431-bp fragment corresponds to the full-length cDNA produced by healthy subjects; the 326-bp transcript also is found in some normal controls and represents an alternative splicing with skipping of exon 2 and conserved frame (GenBank AJ005891); in the 187-bp transcript skipping of exons 2 and 3 was seen, resulting in a frame shift. The putative resulting protein has an abnormal amino-acid composition and it is truncated owing to a premature stop codon in exon 7.

Patient 2 is hemizygous for a C→T transition in exon 3 at nt323, causing the substitution of a threonine with methionine at position 108 (T108M). This missense mutation affects the proline-rich domain and the resulting protein has a predicted normal forkhead domain. The mutations of the 2 patients were not found in the screening of the 200 healthy adult men.

Phenotype and Function of Regulatory T Cells

The 2 IPEX patients had a CD4⁺ T-cell subpopulation that constitutively expressed CD25. Flow cytometry analysis showed that the percentage of CD4⁺CD25⁺ was normal compared with that found in healthy controls (Table 1). Children with other chronic autoimmune diseases who were treated with the same immunosuppressants had similarly normal percentages of CD4⁺CD25⁺ T cells (Table 1). Treg cells are enriched in the population, expressing high levels of CD25 (CD4⁺CD25⁺^{bright} T cells). Evaluation of CD4⁺CD25⁺^{bright} T cells showed no differences from healthy controls (Table 1). In both IPEX patients, similar to disease/treatment control patients, a negligible percentage of the CD4⁺CD25⁺ T cells expressed the activation marker CD69 (Table 1), therefore suggesting that antigen-activated T cells did not contribute significantly to the subset of CD4⁺CD25⁺ T cells.

To investigate the function of the CD4⁺CD25⁺ population, we evaluated the ability of CD4⁺CD25⁺ to suppress the proliferation of autologous CD4⁺CD25[−] T cells. CD4⁺CD25⁺ T cells from healthy controls suppressed the proliferation of autologous effector T cells by 15%–40% (Figure 3). On the contrary, coculture of CD4⁺CD25⁺ T cells from IPEX patients with autologous T cells did not result in any suppression, but rather in increased proliferation (Figure 3). CD4⁺CD25⁺ T cells from disease/treatment controls suppressed proliferation of autologous T cells in a range similar to that of healthy controls, excluding that the lack of suppression

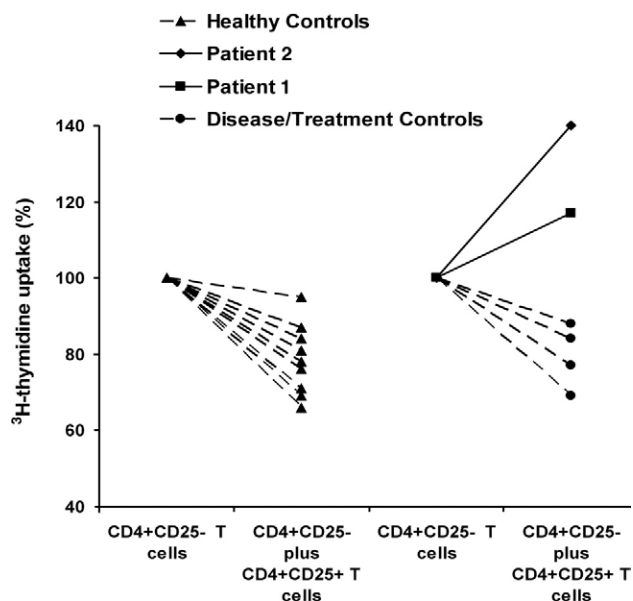


Figure 3. Proliferation of effector CD4⁺CD25⁻ T lymphocytes in the absence or in the presence of CD4⁺CD25⁺ Tregs from healthy controls, from autoimmune/treatment controls, and from the 2 IPEX patients. CD4⁺CD25⁻ and CD4⁺CD25⁺ populations were purified by sorting and were stimulated with anti-CD3 and anti-CD28 as described in the Materials and Methods section. Results are expressed as a percent of thymidine uptake of stimulated effector CD4⁺CD25⁻ T lymphocytes cultured in the absence of CD4⁺CD25⁺ T cells. ▲, Healthy controls; ◆, patient 2; ■, patient 1; ●, disease/treatment controls.

by the CD4⁺CD25⁺ cells of IPEX patients was secondary to treatment.

Discussion

The course of our patients appears to be milder than that of other IPEX patients reported. Both of our patients survived the early years of life and patient 1 already has reached young adulthood. In both of our patients early onset severe enteropathy was the prominent clinical feature. In addition, our patients did not develop clinical or subclinical (presence of autoantibodies) evidence of T1D or other autoimmune endocrinopathies, or severe or unusual infections despite chronic immunosuppression.

In patient 1 the intestinal biopsy examination showed subtotal villous atrophy, an intraepithelial lymphocyte count greater than 40%, and mononuclear cell infiltrate. This was classified as stage 3a, according to the modified Marsh classification,¹¹ and the persistently high titers of antigliadin and antiendomysial antibodies were consistent with a diagnosis of celiac disease. However, patient 1 did not respond to a gluten-free diet. Whether he is affected with both IPEX and celiac disease, or whether the features compatible with celiac disease are manifes-

tations of the immune dysregulation of IPEX, is difficult to ascertain. From a practical point of view, this case indicates that in patients with early onset enteropathy and celiac disease-related antibodies, a failure to respond to a gluten-free diet should prompt the suspicion of IPEX syndrome, even in the presence of histologic findings highly suggestive of celiac disease.

To date, the majority of FOXP3 mutations reported in IPEX patients are either missense mutations in the forkhead DNA binding domain, or deletions resulting in an altered, truncated, or missing forkhead domain.^{1,4} In patient 2, a missense mutation in exon 3 leads to an amino-acid substitution that does not affect the forkhead domain. In patient 1, the mutation close to the splice site generates abnormal and normal transcripts, suggesting that at least a certain amount of normal FOXP3 protein is produced. This phenomenon (ie, splicing mutations resulting in abnormal and some normal transcripts), not reported in IPEX, already has been described in X-linked adrenoleukodystrophy.¹² Therefore it is tempting to speculate that a milder IPEX phenotype may be associated with FOXP3 mutations that do not abrogate the function of the forkhead domain. Further studies of genotype/phenotype correlation are needed. Interestingly, the only FOXP3 mutation associated with a mild IPEX phenotype reported to date is that of the family described by Powell et al,² which affected the first polyadenylation signal, possibly resulting in messenger RNA destabilization and in reduced production of a normal protein.¹³

In mice, FOXP3 has been shown to be essential for the development and function of Treg cells. No data are available on the function of Treg cells in the patients with IPEX. The percentage and absolute number of circulating CD4⁺CD25⁺ T cells were in the low-normal range in both our patients. Because only a negligible percentage of these cells expressed the activation marker CD69, these CD4⁺CD25⁺ T cells are unlikely to represent activated T cells. The number of CD4⁺CD25⁺ has been reported only in 1 patient with IPEX.^{2,14} In this case CD4⁺CD25⁺ were found to be within the normal range; however, increased expression of activation markers suggested that they were antigen-activated T cells.¹⁴ Methodologic difference (activation markers used) or difference in the age of the patients (ie, there was only 1 patient in Bakke et al¹⁴ and he was followed-up until age 18 months) could account for this apparent discrepancy. We found that the CD4⁺CD25⁺ T cells of both our patients were unable to perform a typical function of Treg cells in vitro: suppression of proliferation of activated T cells. Because anergy (absence of response to

T-cell receptor cross-linking) is another feature of cultured Treg cells,¹⁵ the increased proliferation in cultures containing CD4⁺CD25⁺ from our IPEX patients suggests lack of anergy. Therefore, the CD4⁺CD25⁺ cells from our patients have a functional impairment, being both unable to suppress and to remain anergic. Taken together, our findings suggest that, even if not affecting the forkhead domain, these novel FOXP3 mutations are relevant clinically and functionally and imply that other domains, besides the forkhead domain, play a role in the FOXP3 function.

In summary, we have reported 2 patients with IPEX presenting with early onset enteropathy without T1D or other endocrinopathies and a mild clinical phenotype. These patients carry novel mutations of the FOXP3 gene that do not abrogate the function of the forkhead domain and have defective Treg cells.

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