

Severe Food Allergy as a Variant of IPEX Syndrome Caused by a Deletion in a Noncoding Region of the *FOXP3* Gene

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Background & Aims: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX; OMIM 304930) syndrome is a congenital syndrome characterized by autoimmune enteropathy, endocrinopathy, dermatitis, and other autoimmune phenomena. In the present work, we aimed to uncover the molecular basis of a distinct form of IPEX syndrome presenting at the edge of autoimmunity and severe allergy. **Methods:** The *FOXP3* gene was sequenced, *FOXP3* messenger RNA (mRNA) was quantified by real-time polymerase chain reaction (PCR), and protein expression in peripheral blood lymphocytes was analyzed by flow cytometry after intracellular staining. In coculture experiments (CD4⁺CD25⁻ and CD4⁺CD25⁺ cells), the functions of regulatory T cells were analyzed. Expression of interferon γ and interleukin 2 and 4 mRNA within the inflamed intestinal mucosa was quantified by real-time PCR. **Results:** Here, we describe a distinct familial form of IPEX syndrome that combines autoimmune and allergic manifestations including severe enteropathy, food allergies, atopic dermatitis, hyper-IgE, and eosinophilia. We have identified a 1388-base pair deletion (g.del-6247_–4859) of the *FOXP3* gene encompassing a portion of an upstream noncoding exon (exon –1) and the adjacent intron (intron –1). This deletion impairs mRNA splicing, resulting in accumulation of unspliced pre-mRNA and alternatively spliced mRNA. This causes low *FOXP3* mRNA levels and markedly decreased protein expression in peripheral blood lymphocytes of affected patients. Numbers of CD4⁺CD25⁺*FOXP3*⁺ regulatory T cells are extremely low, and the CD4⁺CD25⁺ T cells that are present exhibit little regulatory function. **Conclusions:** A new mutation within an upstream noncoding region of *FOXP3* results in a variant of IPEX syndrome associating autoimmune and severe immunoallergic symptoms.

An old but initially speculative immunologic concept claiming the existence of suppressor T lymphocytes^{1,2} was substantiated with the discovery that mutations of the transcription factor *FOXP3* result in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome^{3–5} and that *FOXP3* is required for the generation of CD4⁺CD25⁺ regulatory T lymphocytes (T_{REG}).^{6–8} A growing body of experimental and in vivo evidence in humans and animals has demonstrated a crucial role for *FOXP3*-expressing CD4⁺ T cells as potent suppressors of self-reactive T-cell activation and proliferation, presumably via direct cell-cell interaction.^{9,10} Some evidence points to a role for T_{REG} cells in the control of immune responses to exogenous antigens, such as dietary food antigens,^{11,12} but much less is known about this aspect of their function.

The *FOXP3* gene maps to Xp11.23 and encodes a 431-amino acid (48 kilodalton) protein, also named *scurfin*. *FOXP3* has significant homology to members of the Forkhead/winged-helix transcription factor family¹³ and plays a central role in the generation of T_{REG} cells. *FOXP3* functions as transcriptional repressor, thus allowing negative control of T-cell activation via DNA sequences containing *FOXP3* binding sites located adjacent to nuclear factor of activated T-cell regulatory sites in cytokine promoters such as interleukin (IL)-2 or granulocyte-macrophage colony-stimulating factor enhancer.^{14–16}

IPEX syndrome is a severe, systemic autoimmune disorder that typically presents in infancy with various autoimmune symptoms including protein-losing enteropathy; early onset, insulin-dependent diabetes mellitus; and other endocrinopathies, dermatitis, and autoim-

Abbreviations used in this paper: IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; T_{REG}, regulatory T lymphocytes.

immune cytopenias.^{17–20} Effective therapeutic options for IPEX syndrome patients are limited. In addition to supportive measures, such as total parenteral nutrition, insulin injections, thyroid hormone substitution, and red blood cell transfusions, various immunosuppressive regimens have been utilized including high-dose steroids, cyclosporin A, tacrolimus, sirolimus, and rituximab with variable success.^{19–23} Because IPEX syndrome results from an absent or dysfunctional lymphocyte subset, bone marrow transplantation offers a potentially curative treatment option for boys suffering from this devastating disorder.^{24–26} Results thus far have been mixed, but efforts are underway to optimize conditioning regimens to improve further this mode of therapy.

Here, we present a kindred with a distinct clinical presentation of IPEX syndrome with no detectable endocrinopathy but an impressive allergic phenotype manifested by severe food allergy and eczema. Molecular analyses revealed a unique *FOXP3* mutation involving a large deletion in an upstream, noncoding region of the *FOXP3* gene. This deletion, encompassing the 3' half of the untranslated –1 exon and approximately 1000 base pairs (bp) of the adjacent intron, leads to markedly reduced *FOXP3* messenger RNA (mRNA) levels and absence of regulatory T cells. These cases provide a unique insight into the critical role of naturally arising CD4⁺CD25⁺FOXP3⁺ T_{REG} cells in controlling immune responses to exogenous antigens as well as in maintaining self-tolerance.

Clinical Features

Patients IV.1 and IV.2

Patient IV.1, (Figure 1) the index case, followed at Necker-Enfants Malades Hospital, Paris, was born in 2000 after an uneventful pregnancy to unrelated, healthy white parents. No abnormalities were observed during the neonatal period while he was exclusively breastfed. At 3 weeks of age, infant formula was introduced, and, within 1 week, he developed massive watery-bloody diarrhea requiring total parenteral nutrition, daily albumin supplementation, and repeated blood transfusions. The severe protein-losing enteropathy was accompanied by the appearance of an erythematous, eczematous skin rash. Laboratory evaluation revealed no signs of glucose intolerance, thyroid abnormalities, Addison's disease, or hematologic abnormalities, and there was no evidence of renal or lung involvement. Immunologic testing at the age of 6 weeks showed normal peripheral blood lymphocyte counts and subsets (3800/μL: CD3⁺, 91%; CD4⁺, 65%; CD8⁺, 21%; CD19, 5%) with reduced natural killer (NK) T cells (CD3⁺CD56⁺, 0.6%) and normal levels of IgG (200 mg/dL), IgM (15 mg/dL), and IgA (26mg/dL). In contrast, IgE levels were strikingly elevated at >3000 μ/mL and associated with eosinophilia (950 cells/μL). At the age of 2 months, high titer antienterocyte antibodies

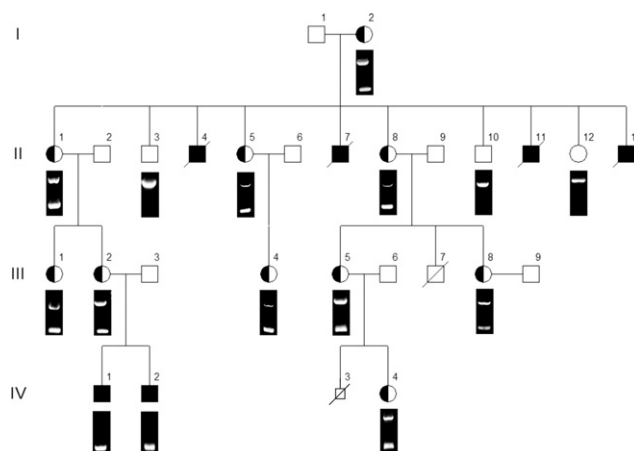


Figure 1. Pedigree demonstrating X-linked inheritance in the identified kindred. Individual family members were tested for the presence of the identified deletion mutation using PCR with forward and reverse primers flanking the deletion in genomic DNA. These primers generate an 1805-base pair fragment from a normal allele and a 417-base pair fragment from an allele harboring the identified 1388-base pair deletion. An ethidium bromide-stained gel demonstrating the fragments amplified from genomic DNA is shown beneath each of the individuals who were tested. Note that the proband (IV.1) and his brother (IV.2) demonstrate only the mutant allele, whereas normal, unaffected males demonstrate only the wild-type allele (II.3 and II.10). All but one of the females tested were found to be carriers of the mutation.

(immunofluorescence study) were demonstrated. Antiautoimmune enteropathy (AIE) 75-kilodalton antibodies²⁷ (directed against intestinal epithelial cell antigens) were initially negative but became strongly positive within the first 4 months of life. No other auto-antibodies were detected, including antinuclear, antiliver, antikidney, antismooth muscle, antimitochondrial, antimicrobial, or antithyroglobulin antibodies. Initial endoscopic evaluation of the gastrointestinal (GI) tract, performed at 7 weeks of age, revealed gastric hyperemia and villous atrophy in the duodenum. Histologic analysis showed an intense lympho-plasmocellular infiltrate with a marked eosinophilic component in the lamina propria of the stomach as well as the duodenum (Figure 2). The epithelial layer was disorganized with a high rate of apoptosis among the enterocytes. Duodenal biopsy specimens were characterized by severe to total villous atrophy and a massive T-cell infiltration in the lamina propria, with no or only moderate increase of intraepithelial lymphocytes. The colonic mucosa revealed superficial ulcerations and a major mononuclear cell infiltrate that was rich in eosinophils. Neither bacteria nor viral pathogens could be identified in the intestinal/colonic mucosa or in peripheral blood.

Patient IV.2, the younger brother of the index case, was born at term in 2002 after an uneventful pregnancy and delivery. Because of the older brother's medical history, the mother's diet was restricted beginning during the third trimester of pregnancy to exclude cow milk protein

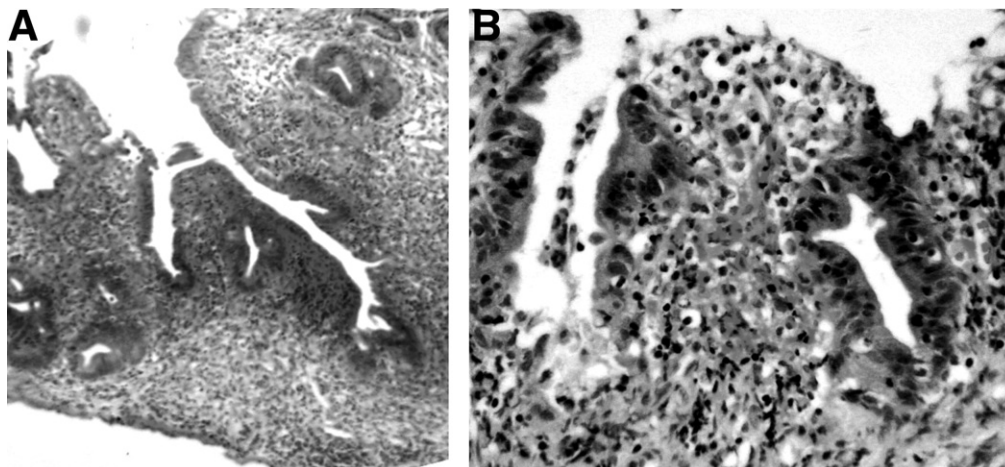


Figure 2. Histologic analysis of a duodenal section of patient IV.1 before treatment. At lower magnification (A; original magnification, 10 $\times$), total to subtotal villous atrophy along with a massive mononuclear infiltration of the lamina propria and severe epithelial alterations can be observed. (B) At higher magnification (original magnification, 40 $\times$), the infiltrate is predominantly of mononuclear origin with few scattered eosinophilic lymphocytes. A crypt abscess of polynuclear lymphocytes is seen in a crypt, which is almost completely destroyed (dedifferentiated epithelial cells and high degree of apoptotic enterocytes). H&E stain.

and other common allergens including eggs, peanuts, and fish. After birth, the patient was exclusively breastfed. Despite these measures, he developed clinical symptoms at 2 months of age, similar to those of his older brother, including eczema and protein-losing enteropathy with severe watery-bloody diarrhea requiring total parenteral nutrition, daily albumin supplementation, and red cell transfusions. There were no signs of glucose intolerance, thyroid dysfunction, or other organ involvement. Immunologic testing at the age of 2 months showed normal peripheral blood lymphocyte counts and subsets (3200/ μ L: CD3 $^{+}$, 90%; CD4 $^{+}$, 64%; CD8 $^{+}$, 20%; CD19 $^{+}$, 6%) with a slightly decreased NK T-cell population (CD3 $^{+}$ CD56 $^{+}$, 2%). IgG, IgM, and IgA levels were normal (300 mg/dL, 38 mg/dL, and 18 mg/dL, respectively). Like the index case, IgE levels were markedly elevated (365 IU/mL at 2 weeks of age) and worsened over time (>2000 IU/mL at 2 months of age) with an accompanying eosinophilia (2400/ μ L). At the onset of symptoms, he was positive for antienterocyte antibodies with pronounced staining along the brush border membrane, anti-AIE 75-kilodalton antibodies, and low titer antismooth muscle autoantibodies. Antinuclear, antithyroglobulin, anti-Langerhans islet, and anti-insulin antibodies were negative.

Both boys (patients IV.1 and IV.2) were treated similarly with a triple-immunosuppressive regimen, consisting of 3 steroid pulses combined with tacrolimus (serum levels, 8–10 ng/mL) and azathioprine (1.5 mg/kg). In each case, the clinical response was prompt, with resolution of the protein-losing enteropathy allowing cessation of parenteral nutrition within 1.5 months. Steroids were tapered and stopped after 3 months. Serum IgE levels decreased but never became normal. All inflammatory parameters (erythrocyte sedimentation rate, C-reactive protein) reverted to normal. After 6 weeks of immunosup-

pressive treatment (prior to oral alimentation), small and large bowel histology of both patients showed normal cytoarchitecture with a marked reduction in the number of inflammatory cells in the lamina propria. Oral alimentation with an elemental diet (NEOCATE; Nutricia, Gaithersburg, MD) was introduced and tolerated by both.

Subsequently, both patients developed multiple skin and food allergies, including erythema, eczema, urticaria, abdominal pain, and watery diarrhea. Allergic skin reactions worsened when oral diversification was started in the older brother (patient IV.1), despite exclusion of all major allergic antigens, such as cow's milk proteins, eggs, peanuts, exotic fruits, or fish. Identical immediate allergic reactions occurred in both boys after the accidental ingestion of minimal traces of cow's milk protein in cookies. These reactions were characterized by immediate vomiting, followed by abdominal pain for 24 hours and liquid stools for 24 hours. In addition, the eczema worsened markedly. In patient IV.1, several episodes of lip swelling, buccal edema, and vomiting were observed upon ingestion of peanut-contaminated chocolate or other food allergens, which could not be identified. Similar episodes occurred in patient IV.2, although less frequently. IgE against cow's milk proteins was repeatedly documented in both children. No bronchopulmonary symptoms were observed except for an episode of *Pneumocystis carinii* pneumonia in patient IV.2, 3 weeks after initiation of immunosuppressive therapy. This resolved with trimethoprim sulfamethoxazol treatment. After switching from tacrolimus to sirolimus combined with azathioprine, gastrointestinal manifestations could be controlled and skin symptoms reduced to mild flare-ups triggered by alimentary allergens. Auto-antibodies (against enterocytes and anti-

AIE 75 kilodaltons) disappeared and have remained negative. Both children are in remission for almost 6 and 4 years, respectively, but show delayed growth and variable eosinophilia.

Patient III.7

Patient III.7 was the second child of healthy parents, born at term in 1971 without any apparent problems. He died at 2 weeks of age of unknown causes and without any evidence of infection or gastrointestinal manifestation. No autopsy was performed. This patient was the maternal uncle of patient IV.3; his mother is a carrier of the FOXP3 mutation.

Patients II.4, II.7, II.11, II.13

All 4 brothers were born at term between 1946 and 1960 to healthy nonconsanguineous parents; the mother is a carrier of the identified FOXP3 mutation. After an initial uncomplicated neonatal period, each child developed symptoms of enteropathy, and all died within the first 2 months of life. Patient II.4, initially exclusively breastfed, died at the age of 5 weeks with severe diarrhea after the introduction of cow's milk. Patient II.7 died at the age of 6 weeks after the onset of intractable diarrhea while still breastfed. Similar clinical symptoms were reported for patients II.11 and II.13, who died at the age of 3 weeks and 8 weeks in 1954 and 1960, respectively. No information is available regarding whether any of these 4 boys presented with additional symptoms, such as endocrinopathy or skin disease.

Other Family Members

A male fetus (patient IV.3) was miscarried at 6 months of pregnancy with no apparent explanation. No morphologic abnormalities were documented, and no necropsy was performed. The mother is a second cousin of patients IV.1 and IV.2, and she and her daughter (patient IV.4) were found to be carriers of the identified FOXP3 mutation.

Ten asymptomatic females spanning 4 generations were found to be heterozygous for the same FOXP3 mutation as present in patients IV.1 and IV.2 (Figure 1). IgE levels were normal, and none of them reported food or cow's milk protein allergy during infancy. In addition, no female carrier suffered from asthma or atopic skin disease, enteropathy, insulin-dependent diabetes, glucose intolerance, or other endocrinopathies.

Using available genomic DNA, a large deletion involving the FOXP3 gene was identified in 12 members of this family. The presence of the deletion only in the affected males (patients IV.1 and IV.2) and not in asymptomatic males (patients II.3 and II.10) strongly suggests that it is pathogenic. It is probable that the 4 brothers presenting with GI symptoms who died within the first 2 months after birth (patients II.4, II.7, II.11, II.13) were also carrying this mutation.

Materials and Methods

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-Paque gradient centrifugation as previously described.⁵

Sequence Analysis of FOXP3

Genomic DNA (gDNA) was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Each of the 11 coding exons of FOXP3 including intron/exon boundaries and the region surrounding the first polyadenylation signal were amplified from gDNA by polymerase chain reaction (PCR) using intronic primer pairs as previously reported.^{5,28} PCR products were separated by 1% agarose gel electrophoresis, purified with the QIAquick PCR Purification Kit (Qiagen) and directly sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Boston, MA).

Rapid genotyping of family members (Figure 1) was accomplished by PCR amplification of fragments from gDNA using primers flanking the identified deletion: forward, 5'-CTCTGTGGTGAGGGGAAGAAATC-3'; reverse, 5'-CATGAATGGCCAATGAATAGTAAAAG-3'. Using these primers, the normal allele yields an amplicon of 1805 bp, and the mutated allele yields an amplicon of 417 bp.

Quantitative Real-Time PCR of FOXP3 mRNA

Total RNA was extracted from 10⁷ fresh PBMCs using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First strand complementary DNA (cDNA) synthesis was performed using the Omniscript RT Kit (Qiagen) per the manufacturer's protocol.

FOXP3 cDNA levels were quantitated using an iCycler iQ Real-Time PCR System (Bio-Rad, Hercules, CA) and Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). Amplification was carried out in a total volume of 20 μ L for 40 to 50 cycles with denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Samples were run in triplicate, and their relative expression was determined by normalizing to expression of the housekeeping gene GAPDH. Values obtained were compared with the level of expression in a normal control sample to calculate the relative fold change. Primers for FOXP3 bind across the spliced junctions of exons 8 and 9 and 9 and 10 distal to the exons affected by the identified mutation: 5'-CTGCCCACACTGCCCTAGTC-3' and 5'-CCATTTGCCAGCAGTGGGTAG-3'. GAPDH primers: 5'-CCACATCGCTCAGACACCAT-3' and 5'-GGCAACAATATCCACTTTACCAGAGT-3'.

Cytokine Profile Within the Intestinal Mucosa

Duodenal biopsy specimens obtained before and 6 weeks after initiation of immunosuppressive therapy were stored in RNAlater (Ambion; Applied Biosystems, Foster City, CA). Total RNA was extracted from the biopsy material using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) and reverse transcribed using Murine-Moloney leukemia virus reverse transcriptase, random hexamers, and oligo dT (Promega, Charbonnières, France) according to standard protocols. In parallel, total RNA was obtained from mucosal biopsy specimens of children with active celiac disease ($n = 5$), Crohn's disease (CD) ($n = 5$), or noninflammatory controls ($n = 5$) as well as 2 additional biopsy specimens of patients IV.1 and IV.2 during follow-up endoscopies. For quantitative real-time PCR, amplifications were performed using Taqman gene assays for interferon (IFN)- γ , IL-2, IL-4, and GAPDH (Applied Biosystems, Paris, France). Forty cycles of amplification were performed with denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute using an ABI PRISM 7700. For IFN- γ , IL-2, and IL-4, results were normalized to the housekeeping gene GAPDH using the $\Delta\Delta C_T$ method after controlling that efficiencies of target and reference amplifications were equal or close to 1.²⁹

Flow Cytometry of FOXP3⁺ CD4⁺CD25⁺ Cells

Surface staining for CD3, CD4, and CD25 was performed on freshly isolated PBMCs. Cells were then washed in phosphate-buffered saline (PBS) and fixed for 30 minutes at 4°C using the eBioscience fixation/permeabilization kit, according to manufacturer instructions. After washing (PBS) and permeabilization (15 minutes at 4°C using the eBioscience permeabilization buffer supplemented with 4% normal rat serum), the cells were incubated with anti-human FOXP3 antibody (clone PCH101, eBioscience) overnight at 4°C. After 3 additional wash steps, the labelled cells were analysed by flow cytometry (BDLaser), gating on CD3⁺ cells.

Coculture Experiments of CD4⁺CD25⁺ and CD4⁺CD25⁻ T Cells

CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were isolated using a CD4⁺CD25⁺ regulatory T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The CD4⁺CD25⁻ cells obtained using this approach were approximately 90% pure, and the CD4⁺CD25⁺ cells were >95% pure as assessed by flow cytometry. Suppressor activity of the CD4⁺CD25⁺ T cells was evaluated using a standard coculture technique with 50,000 CD4⁺CD25⁻ effector cells and 10,000 CD4⁺CD25⁺ regulatory T cells per well in the presence of plate-bound anti-CD3 antibody (UCHT1 1 μ g/mL; Beckmann Coulter, France). After 5 days of culture, cells were pulsed with H³-thymidine for 18 hours,

and DNA synthesis was estimated as previously described.³⁰ Suppressor activity of the CD4⁺CD25⁺ cells was determined as the difference in the proliferation rate observed when anti-CD3-stimulated CD4⁺CD25⁻ T cells

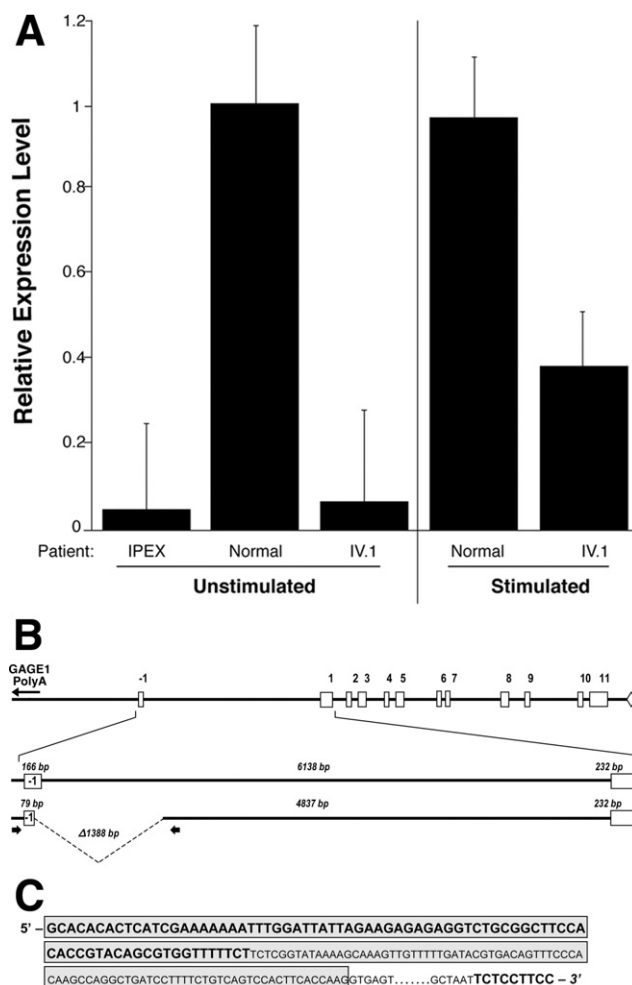


Figure 3. (A) FOXP3 mRNA levels are low in the proband. FOXP3 mRNA levels in fresh or PHA/IL-2 stimulated PBMCs were quantified using quantitative real-time PCR. Expression levels were normalized to the housekeeping gene GAPDH. Data are shown as relative expression with the control normalized to an expression level of 1.0. Error bars show the standard deviation of 3 independent runs. The expression level in the proband (IV.1) is shown relative to a normal control and a patient with IPEX syndrome who harbors a mutation in the polyadenylation site of the FOXP3 gene that leads to mRNA instability.³¹ (B) Genomic structure of the human FOXP3 locus demonstrating the position of the identified deletion. The position of the protein coding exons (1–11) are shown relative to the single upstream noncoding exon (–1) and the transcriptional start site and direction of the next upstream gene, GAGE1. The region containing exons –1 and 1 with the intervening intron is expanded to show the location of the identified 1388-bp deletion mutation (represented by the dashed line). The arrows flanking the deletion show the locations and directions of the primers used to amplify the fragments that allowed rapid screening of family members (Figure 1). (C) Genomic sequence of the identified deletion mutation. The deleted fragment is shown in small font with the remaining flanking genomic DNA sequence shown in large bold font. The extent of exon –1 is shown within the shaded box demonstrating that the deletion includes the 3' half of exon –1 including the splice donor site.

were cultured alone or in the presence of the CD4⁺CD25⁺ T cells. This study was approved by the local ethics committee, and both parents as well as all involved family members gave written consent for these analyses.

Results

Genetic Analysis

The human *FOXP3* gene consists of 11 protein-coding exons (exons 1–11) as well as a noncoding exon (exon –1) located 6 kilobases (kb) upstream of exon 1 (Figure 3). All mutations of the *FOXP3* gene identified to date are located within, or adjacent to, one of the 11 translated exons or within the first polyadenylation site. Based on the clinical phenotype observed in affected members of this family, we sequenced exons 1–11 of the *FOXP3* gene, including all intron/exon boundaries and the first polyadenylation site in patient IV.1 but found no mutation.

The clear X-linked inheritance pattern in the setting of an IPEX-like phenotype suggested the possibility of a defect in a regulatory region of the *FOXP3* genomic locus that might lead to decreased expression of the gene product. To evaluate this possibility, we quantitated *FOXP3* mRNA levels in PBMC from patient IV.1 using gene-specific primers and real-time PCR. This patient was found to have expression levels comparable with a known IPEX syndrome patient whose mutation in the first polyadenylation site leads to mRNA instability and rapid mRNA degradation³¹ (Figure 3A). Both were significantly low relative to the normal control (5%–7% of normal) (Figure 3A).

FOXP3 expression is limited almost exclusively to the CD4⁺*FOXP3*⁺ regulatory T-cell population in normal PBMC. Because the CD4⁺*FOXP3*⁺ regulatory T-cell population is markedly diminished or absent in many patients with IPEX syndrome,³² low *FOXP3* mRNA expression may reflect a paucity of *FOXP3*-expressing cells and not actually low mRNA expression on a per cell basis. To address this question, *FOXP3* mRNA was also quantified in PBMC stimulated with phytohemagglutinin (PHA) (10 μg/mL) and IL-2 (100 U/mL). We and others have demonstrated that activation of human PBMC leads to increased expression of *FOXP3* in most CD4⁺ and CD8⁺ T cells.^{32–35} In PBMC from both patient IV.1 and the normal control, activation led to increased *FOXP3* mRNA expression (data not shown), but levels in the patient's cells remained persistently low relative to the normal control (approximately 40%) (Figure 3A). The observed increase in *FOXP3* message with activation of the patient's cells suggested that at least some of the sequences required for inducible mRNA expression were present. It did not, however, rule out the possibility that some regulatory sequences required for optimal expression were altered or that mRNA stability was affected.

To investigate the possibility of a defect in an upstream, noncoding region of the *FOXP3* gene, we amplified and sequenced approximately 10-kb upstream of the first coding exon (exon 1) to the transcriptional start site of the next encoded gene, *GAGE1* (Figure 3B). This revealed a 1388-bp deletion (g.del-6247_–4859) (numbering per den Dunnen and Antonarakis³⁶) encompassing the 3' half of the untranslated exon –1 and extending downstream more than 1 kb into the adjacent intron (Figure 3C). This novel mutation was also present in patient IV.2 but not in more than 100 normal X chromosomes or in clinically normal males (patients II.3 and II.10) from this kindred (data not shown).

The large deletion made it possible to develop a rapid PCR-based screening test to evaluate quickly other members of this kindred and to identify carrier females. Specific primers were designed to regions flanking the deletion to amplify a product of 1805 bp from the normal allele and a product of 417 bp from the mutated allele. As noted in Figure 1, the 2 affected males IV.1 and IV.2 demonstrate only the 417-bp product, whereas clinically normal males (patients II.3 and II.10) demonstrate only the larger 1805-bp product. In carrier females, both products are generated. Interestingly, all but 1 female (patient II.12) in this 4-generation kindred were found to be carriers (Figure 1).

Effect of the Identified Mutation on *FOXP3* Synthesis

The large deletion identified in this kindred encompasses the 3' half of exon –1 and a portion of the adjacent intron leading to absence of the canonical splice donor site at the 3' end of exon –1 (Figure 3B and C). We predicted that this deletion may prevent splicing of the *FOXP3* pre-mRNA. To evaluate this possibility, PCR primers (PS 1) were designed in the 5' portion of exon –1 and spanning the exon/intron junction at the 5' end of exon 1 (Figure 4B). These primers amplify a product only if unspliced pre-mRNA containing intron –1 is present. This primer pair will not amplify a product from spliced mRNA lacking intron –1. PCR amplification using these primers and cDNA from a normal control yielded no detectable product, suggesting that there is little unspliced pre-mRNA under normal conditions. Amplification from the cDNA of patient IV.1 yielded a 4880-bp fragment containing the upstream portion of exon –1 and the remaining portion of intron –1 lacking the deleted fragment (Figure 4A and data not shown). This suggests that, in the patient, there is decreased efficiency of mRNA splicing leading to accumulation of unspliced pre-mRNA that may be degraded via nonsense-mediated decay.³⁷

Because the identified deletion included only upstream sequences of the *FOXP3* gene, it was theoretically possible that the patient could generate wild-type protein if protein coding exons 1–11 were spliced normally. To deter-

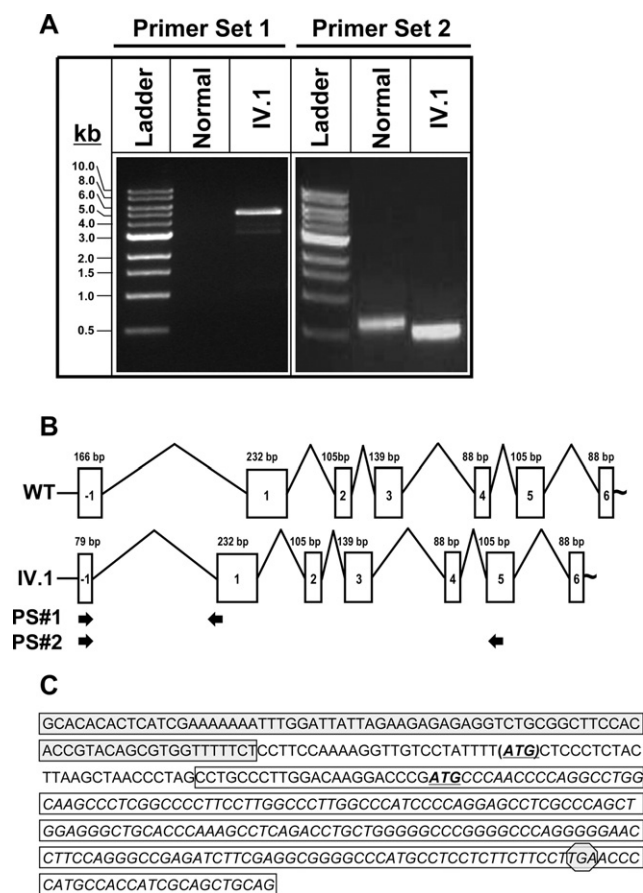


Figure 4. The identified deletion mutation leads to abnormal mRNA splicing. (A) Ethidium bromide-stained agarose gel showing *FOXP3* cDNA fragments amplified from a normal control and patient IV.1. Primer set 1, capable of amplifying only unspliced pre-mRNA, demonstrates amplification of a 4880-bp fragment in patient IV.1 but not in the control (left panel). Primer set 2, which amplifies spliced mRNA, demonstrates amplification of a 793-bp fragment from the normal control and a 601-bp fragment from patient IV.1 (right panel). In each case, amplification of the fragment from patient IV.1 required the use of 10-fold more template than the normal control and an increase in PCR amplification cycles from 30 to 40. (B) Structure of the wild-type and mutant *FOXP3* pre-mRNA. The position of primer set 1 (PS 1) and primer set 2 (PS 2) are shown. (C) Sequence of the 601-bp spliced mRNA fragment from patient IV.1. The remaining portion of exon -1 is shown in the shaded box, and exon 1 is shown in the unshaded box with the usual translation initiation codon shown in bold italics within exon 1. The fragment in the center is derived from the intronic region downstream of the deletion, and it is spliced to exon 1 via an alternative splice donor site. The premature translation initiation codon within this intronic fragment is shown in parentheses and bold italics. Initiation from this alternative, upstream ATG ends in a stop codon in exon 1, denoted by the shaded octagon.

mine whether the patient could generate any correctly spliced *FOXP3* mRNA product, PCR primers (PS 2) were designed in the 5' portion of exon -1 and in exon 5 (see Figure 4B). PCR amplification from the cDNA of a normal control readily generated the predicted 793-bp fragment corresponding to fully spliced mRNA including exons -1 through exon 5 (Figure 4A). Because of the relatively low *FOXP3* mRNA expression levels in the pa-

tient's sample, amplification from the cDNA of patient IV.1 was more difficult and required the use of at least 10-fold higher cDNA template concentrations and an increase in PCR amplification cycles from 30 to 40. With these modifications, a 601-bp fragment was amplified and subsequently sequenced. This product represented spliced mRNA containing the remaining portion of exon -1 appended to a 52-bp intronic fragment derived from the sequence immediately downstream of the deletion. The 3' end of this intronic fragment was spliced via an alternate splice donor site to an intact exon 1 (see Figure 4C). The inserted intronic fragment contained a new ATG translation initiation codon. Initiation of translation from this alternative, upstream ATG would yield an out-of-frame polypeptide of 77 amino acids that terminates with a premature stop codon near the 3' end of exon 1 (Figure 4C). A second premature stop codon in this reading frame would be encountered in exon 3. Nonsense codons encountered upstream of spliced exon-exon junctions can target mRNA molecules for rapid degradation via nonsense-mediated decay mechanisms.^{37,38} Whether this may explain the low *FOXP3* mRNA levels observed in PBMC from affected males in this kindred remains to be determined. Interestingly, the spliced product from the patient also lacked exon 2, a well-described, naturally occurring splice variant of the *FOXP3* mRNA.³⁹

Evaluation of Regulatory T-Cell Number and Function

To determine whether the low *FOXP3* mRNA expression in PBMC correlated with decreased *FOXP3* protein expression and consequently decreased regulatory T-cell numbers, PBMC were stained with specific antibodies to CD3, CD4, CD25, and *FOXP3* and evaluated by flow cytometry. Five percent to 6% of the CD4⁺ T cells from normal controls demonstrated *FOXP3* expression (Figure 5A), consistent with previous reports.^{32,40} Virtually all *FOXP3*⁺ cells from the normal control were CD25^{high} (data not shown). In contrast, less than 1% of the CD4⁺ cells in patient IV.1 and IV.2 were *FOXP3*⁺, with none being *FOXP3*^{high} (Figure 5A). Interestingly, despite the low number of *FOXP3*⁺ cells, the number of CD4⁺CD25⁺ cells was comparable with healthy controls in both patients (data not shown), suggesting that these cells are either regulatory T cells that lack *FOXP3* expression or are activated T cells expressing CD25 as a marker of their activation.

To evaluate whether the CD4⁺CD25⁺ cells present in the patients have regulatory function, a standard coculture assay was performed.³⁰ As shown in Figure 5, CD4⁺CD25⁺ T cells from a normal control and from 2 affected males (patients IV.1 and IV.2) did not proliferate significantly in response to cross-linking of CD3 (Figure 5B). In contrast, CD4⁺CD25⁻ effector cells proliferated strongly in response to anti-CD3 stimulation, with H³

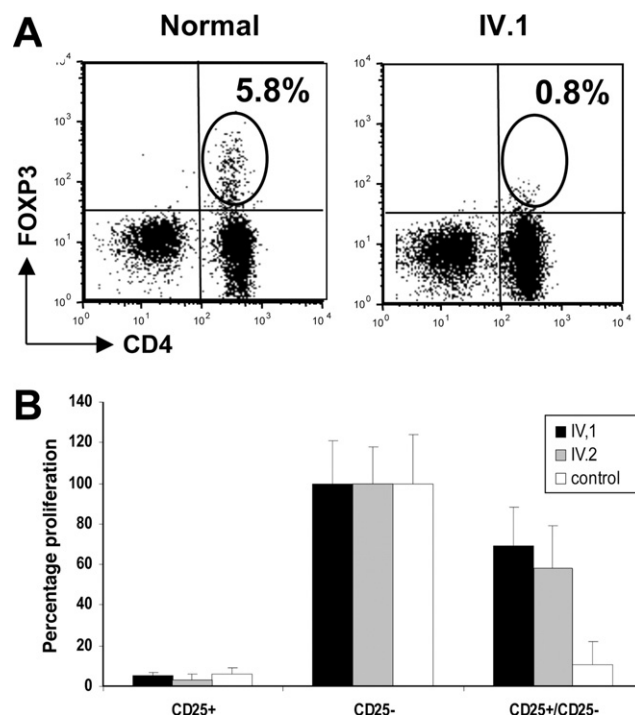


Figure 5. (A) FOXP3 protein expression in peripheral blood mononuclear cells. PBMC from patient IV.1 and a healthy control were stained intracellularly for FOXP3 and analyzed by flow cytometry for the presence of CD4⁺CD25⁺FOXP3⁺ cells. Gating on CD3⁺ cells, the normal control patient showed expression of FOXP3 protein in approximately 6% of the cells. In contrast, FOXP3 expressing cells were almost undetectable in patient IV.1. (B) CD4⁺CD25⁺ T cells from patients IV.1 and IV.2 demonstrate decreased suppressive activity. The suppressive activity of CD4⁺CD25⁺ T cells from patients and normal controls was tested in a coculture system using separated CD4⁺CD25⁻ effector cells and CD4⁺CD25^{high} T cells. The purified effector cells (CD4⁺CD25⁻) underwent significant proliferation upon CD3 cross-linking, whereas the purified CD4⁺CD25^{high} cells showed little proliferative response. In coculture, the CD4⁺CD25^{high} population derived from patients IV.1 and IV.2 demonstrated markedly diminished suppressive activity compared with cells derived from a healthy normal control patient.

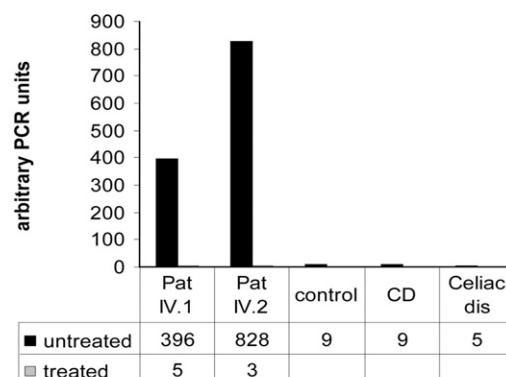
thymidine incorporation being slightly higher in control lymphocyte cultures compared with those of patients IV.1 and IV.2 (Figure 5B and data not shown). This is likely the result of exposure to immunosuppressive medications that each of the patients was taking at the time. Coculture of CD4⁺CD25⁺ regulatory T cells from the normal control with autologous responder cells demonstrated the highly suppressive effect of the CD4⁺CD25⁺ regulatory T cells (90% suppression). In contrast, the suppressive activity of CD4⁺CD25⁺ T cells from patients IV.1 and IV.2 was found to be markedly reduced compared with the normal control (34% and 41%, respectively), indicating altered regulatory function (Figure 5B).

Cytokine Expression Profile Within the Intestinal Mucosa

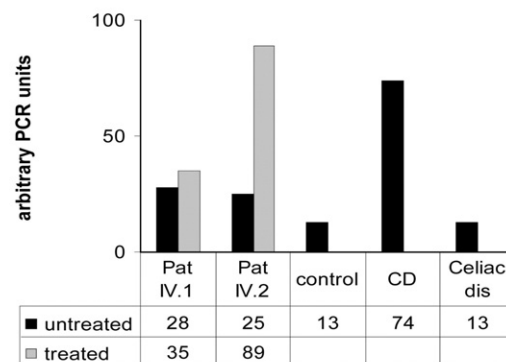
Because of the dramatic allergic phenotype in these IPEX syndrome patients, analysis of the cytokine

expression profile within inflamed duodenal mucosa was performed to evaluate whether the infiltrate was more characteristic of a T helper (Th) 1 or Th2 response. Biopsy specimens were obtained from patients IV.1 and IV.2 before and 6 weeks after initiation of immunosuppression (steroids, tacrolimus, and azathioprine). Cyto-

IL-4



IL-2



IFN γ

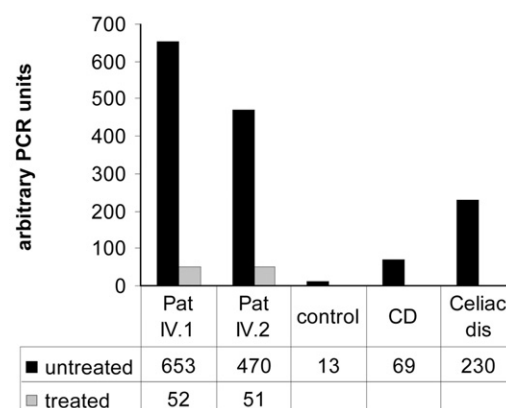


Figure 6. Real-time PCR analysis of cytokine expression profile in inflamed duodenal tissue. Th1 (IFN- γ) and Th2 (IL-4) as well as regulatory cytokine (IL-2) expression were analyzed before and after successful therapy in patients IV.1 and IV.2 compared with normal noninflammatory controls (n = 5) and inflammatory controls from patients with active Crohn's disease (CD, n = 5) or untreated celiac disease (n = 5). Inflamed duodenal mucosa from patients IV.1 and IV.2 demonstrates a tremendous up-regulation of IL-4 and IFN- γ , which returns to control levels following successful immunosuppressive therapy. In contrast, IL-2 expression is modest in untreated patients but increases with therapy.

kine mRNA expression levels in the mucosal biopsy material were evaluated by semiquantitative and quantitative PCR. Prior to treatment, patients IV.1 and IV.2 had markedly increased levels of IFN- γ and IL-4 transcripts compared with the normal control patients (Figure 6), suggesting a mixed Th1 and Th2 cell infiltrate in the bowel mucosa. With therapy, IFN- γ and IL-4 levels returned to near normal (Figure 6). In comparison, duodenal biopsy specimens from patients with other active inflammatory bowel diseases, including celiac disease and Crohn's disease, demonstrated very little IL-4 mRNA expression but increased levels of IFN- γ mRNA, suggesting primarily Th1-mediated bowel inflammation in these diseases. Although IFN- γ is only modestly up-regulated in Crohn's disease, it is strongly up-regulated in celiac disease (Figure 6). Despite this, the mean level of IFN- γ expression in the 2 untreated IPEX syndrome patients (>550 arbitrary units) was more than 2-fold higher than in 5 active celiac disease patients, likely reflecting the intensity of inflammation in patients with untreated IPEX syndrome. Interestingly, IL-2 mRNA expression levels were significantly increased in bowel mucosa from patients with untreated Crohn's disease but were only increased in IPEX syndrome patients following immunosuppressive therapy (Figure 6). As a control, IL-5 mRNA expression was also evaluated in the mucosal samples but was absent in both IPEX syndrome and the other inflammatory bowel conditions tested (data not shown).

Discussion

The family reported here presented with an atypical form of IPEX syndrome that was characterized by multiple severe food allergies associated with AIE. The patients also had eczema, hyper-IgE, and eosinophilia but, unlike most other cases of IPEX syndrome, did not present with endocrinopathy or cytopenia. The restricted symptomatology observed in the affected members of this family demonstrates the clinical heterogeneity of the IPEX syndrome and expands the range of patients for whom IPEX syndrome should be considered as a diagnosis. As suggested by this study, infants presenting with an unusually severe form of allergy early in life may require screening for IPEX syndrome. To date, no clear genotype-phenotype correlations have been reported, but atypical or milder forms of IPEX syndrome have been observed, and long-term survivors such as members of this family have been described.^{17,22,41,42} Similar to other reports,^{31,43} all of the female carriers identified in this kindred were healthy and had no signs of autoimmunity. Interestingly, inheritance of the mutant allele in this family appears to be skewed in the female offspring of carrier mothers (see Figure 1); 90% (9/10) of females compared with 60% (6/10) of the male offspring inherited the mutant allele. Although it is theoretically possible that gametes carrying the mutant allele are more successful at fertilization or more viable than their wild-type counterparts, there is

no evidence that FOXP3 is expressed at any time during fertilization or development. We believe that this difference is most likely a chance occurrence. This is supported by the inheritance observed in a very large kindred with IPEX syndrome initially described by Powell et al¹⁷ and Bennett et al.³¹ Currently in this kindred, 65.2% (15/23) of females born to carrier mothers have inherited the mutant allele, and 60.5% (23/38) of males have the mutation (data not shown). These numbers are consistent with the 60% rate of inheritance seen in the males of the kindred presented here and are close to the expected frequency of 50%.

Sequence analysis revealed a novel mutation in an upstream noncoding region of the *FOXP3* gene (Figure 3B). The mutation consists of a large deletion involving the 3' half of the untranslated exon -1 and more than 1 kb of intron -1 of the *FOXP3* gene, resulting in the loss of 1388-bp of genomic DNA. Because the deletion affects only the untranslated portion of the gene, it is theoretically possible that the patients could express normal FOXP3 mRNA and protein. Unfortunately, the deletion leads to loss of the usual splice donor site at the 3' end of exon -1 and, consequently, decreased efficiency of pre-mRNA splicing as evidenced by the accumulation of unspliced pre-mRNA (Figure 4A). The only spliced mRNA product amplified from the patients included an insertion of a 52-bp intronic fragment from intron -1. This intronic fragment introduces a new translation initiation codon upstream of the endogenous ATG that would generate a 77-amino acid out-of-frame polypeptide terminating in a premature stop codon within exon 1. Both the unspliced and alternatively spliced mRNA products are predicted to meet criteria for rapid degradation via nonsense mediated decay mechanisms,³⁷ although this has not been directly tested.

Functional analyses using a coculture system of CD4⁺CD25^{high} T cells with CD4⁺CD25⁻ effector cells revealed a marked lack of suppressor function by CD25^{high} T cells from both patients compared with normal controls. It is important to note that both patients IV.1 and IV.2 were under tacrolimus-based immunosuppression at the time of analyses. Tacrolimus might impact on the function of regulatory T cells. However, control experiments with lymphocytes from tacrolimus-treated patients indicate that the effect on T-cell proliferation is extremely weak under the experimental conditions used in this study (data not shown). A second important point in the interpretation of the functional data is the fact that other FOXP3-negative T cells with regulatory functions exist, such as Th3 or Tr1 cells, characterized by the production of transforming growth factor β and IL-10, respectively.⁴⁴ To date, it is not really clear of what origin these cells are and what their respective regulatory roles are.

CD4⁺CD25^{high} cells were present in both patients at levels similar to healthy donors, and FOXP3 protein was

not detectable in this subset of T cells. These findings suggest that this mutation, involving a region upstream of the coding exons, results in the absence of functional FOXP3-expressing regulatory T cells. As a consequence, uncontrolled T-cell responses occurred, exemplified by the high levels of IFN- γ and IL-4 mRNA expression by cells infiltrating the inflamed bowel mucosa in the untreated patients (Figure 6).

The simultaneous activation of both Th1 and Th2 responses in patients IV.1 and IV.2 prior to immune suppression seems plausible in light of the clinical picture of autoimmune GI disease (Th1 mediated) and severe food allergies (Th2 mediated). Under normal conditions, however, Th1 and Th2 T-cell responses are thought to be mutually exclusive, suggesting that in the case of IPEX syndrome, absence of FOXP3⁺ T_{REG} cells permits global activation of all effector T-cell populations. The importance of FOXP3⁺ T_{REG} in the control of Th1 effector cell activity and the maintenance of tolerance to self antigen has been established⁴⁵ and is exemplified in our patients by the observation that IFN- γ levels in the inflamed intestinal mucosa prior to treatment were more than 2-fold higher than those observed in active celiac disease, which is itself characterized by a massive Th1, IFN- γ -mediated immune response (Figure 6). A role for regulatory T cells in the control of Th2 responses and tolerance to oral antigens has been postulated for some time.^{46,47} Recent murine studies have demonstrated that CD4⁺CD25⁺ T_{REG} cells are required for the induction of oral tolerance^{48,49} and that administration of oral antigen dramatically induces antigen-specific CD4⁺CD25⁺ T_{REG} cell numbers and function.^{50–52} Some evidence suggests that, in addition to the usual thymus-derived T_{REG} population, the antigen-specific T_{REG} cells induced by oral antigen may be derived in the periphery from CD4⁺CD25[–] T cells.⁵³ Karlsson et al¹¹ have recently shown in humans that resolution of cow's milk allergy is associated with increased numbers of circulating CD4⁺CD25⁺ T cells and decreased in vitro proliferative responses of bulk PBMCs to bovine β -lactoglobulin. The in vitro proliferative responses to β -lactoglobulin increased more than 4-fold when CD25⁺ cells were depleted from the PBMCs, suggesting a direct link between the increase in CD4⁺CD25⁺ T_{REG} cells and resolution of the cow's milk allergy.¹¹

The inflammatory mucosal lesions observed in both patients showed a combination of severe villous atrophy and massive mucosal mononuclear cell infiltration (Figure 2). This infiltrate consists predominately of T lymphocytes and is a main feature of AIE. Most often, villous atrophy is associated with crypt hyperplasia.⁵⁴ Total villous atrophy on duodenal biopsies can initially mislead to suspect celiac disease. However, in celiac disease, villous atrophy is associated with a striking increase in the number of intraepithelial lymphocytes.⁵⁵ In contrast, T-cell infiltration in AIE is predominantly observed in the

lamina propria, with no or only moderate increase in intraepithelial lymphocytes.⁵⁶ Whereas in celiac disease a marked increase in T lymphocytes expressing the T-cell receptor of the γ/δ^+ type was observed,⁵⁷ in AIE, T-cell receptor expression is restricted to the α/β^+ subset. In some patients with AIE, villous atrophy is associated with massive epithelial cell apoptosis and crypt abscess formation, as seen in our patients (Figure 2).

Auto-antibodies of the antienterocyte or anticolonyocyte type are a hallmark of AIE, and these antibodies are present in the vast majority of IPEX syndrome patients.^{58,59} Antienterocyte antibodies are most often of IgG origin, but auto-antibodies of the IgA or IgM isotype were also described in a subgroup of patients.⁶⁰ Indirect immunofluorescence studies showed that these auto-antibodies are directed against components of the cytoplasm of mature enterocytes, with an increasing intensity toward the villus tip. Positive staining of the intestinal brush border membrane can also be observed. A pathogenetic role in the onset of intestinal inflammation—as suspected in the past—is unlikely. They are rather an epiphenomenon of intestinal inflammation. A precise kinetic study performed in one patient showed that auto-antibodies occurred after the onset of intestinal lesions.⁶¹ Another, highly specific antibody often detected in IPEX syndrome patients is directed against a gut- and kidney-specific 75-kilodalton antigen named AIE75.^{27,62} This antigen shares over 99% identity with NY-Co-38, a colon cancer-related autoantigen.⁶³

Interestingly, in 6 of the 8 affected boys in this family, the onset of severe diarrhea occurred within the first 8 weeks of life. A major change in the type of alimentation, such as switching from mother's milk to a cow's milk-based infant formula preceded clinical symptoms in some of the patients. In patients IV.1 and IV.2, this was associated with the appearance of specific IgE at very high titers against cow's milk protein (F2 RAST highly positive, data not shown). A marked worsening of symptoms has been reported in other patients with IPEX syndrome following immune activation by either immunization or infections.¹⁷ Additionally, Zahorsky-Reeves and Wilkinson⁶⁴ have recently shown that scurfy mice whose T cells bear a monospecific T-cell receptor (Rag1^{–/–} sf/Y Ova) require antigen (ovalbumin) exposure to trigger scurfy disease and unmask the regulatory T-cell defect. Similar to ovalbumin in this mouse model, antigenic epitopes from cow milk proteins appear to initiate immune activation in these patients and precipitate progression of disease. It is not known whether the same antigen is capable of simultaneously inducing both Th1 and Th2 responses or if different antigens are required.

The prominence of GI and skin disease in IPEX syndrome and particularly in the patients reported here suggests an important role for T_{REG} cells at interfaces that are unrelentingly exposed to exogenous antigens

such as bacterial products and alimentary antigens. Recent data suggest that CD4⁺CD25⁺ T_{REG} cells express receptors of the innate immune system including Toll-like receptors 2, 4, 5, 7, and 8, enabling them to sense bacterial products.^{65,66} In vitro stimulation of purified murine CD4⁺CD25⁺ T cells by activation of Toll-like receptor 4 with bacterial lipopolysaccharide increased the suppressor efficiency of these cells,⁶⁵ whereas activation of Toll-like receptor 2 decreased the suppressive activity.⁶⁶ Ongoing studies to clarify further the precise role of T_{REG} cells at these important self/environment interfaces will help to elucidate the pathogenesis of IPEX syndrome and may provide opportunities for more targeted therapeutic interventions.

In summary, IPEX syndrome is a more heterogeneous disorder than previously recognized, exemplified by this family demonstrating a form of the syndrome combining autoimmunity and severe allergy. We were able to demonstrate in our patients that a large deletion in an upstream noncoding region of FOXP3 gene causes a defect in mRNA splicing. Subsequently, FOXP3 mRNA and protein levels are almost undetectable, causing profound T_{REG} dysfunction. This presents clinically as a combined Th1/Th2 disorder, as exemplified by the cytokine mRNA profile within the inflammatory intestinal mucosa. This observation gives new insight into the physiopathology of immunoallergic dysregulation, opening new diagnostic and potentially therapeutic avenues.

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