CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked–like syndrome, and defective IL-10 expression from CD4 lymphocytes

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Background: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) results in systemic autoimmunity from birth and can be caused by mutations in the transcription factor forkhead box P3 (FOXP3).
Objective: To determine if Foxp3 is required for the generation of IL-10–expressing T regulatory cells.
Methods: CD4 lymphocytes were isolated from patients with IPEX-like syndromes and activated with antibodies to CD3 and CD46 to generate IL-10–expressing T regulatory cells.
Results: We describe a patient with clinical manifestations of IPEX that had a normal Foxp3 gene, but who had CD25 deficiency due to autosomal recessive mutations in this gene. This patient exhibited defective IL-10 expression from CD4 lymphocytes, whereas a Foxp3-deficient patient expressed normal levels of IL-10.
Conclusion: These data show that CD25 deficiency results in an IPEX-like syndrome and suggests that although Foxp3 is not required for normal IL-10 expression by human CD4 lymphocytes, CD25 expression is important.
Clinical implications: Any patient with features of IPEX but with a normal Foxp3 gene should be screened for mutations in the IL-2 receptor subunit CD25. (J Allergy Clin Immunol 2007;119:482-7.)

Key words: *IPEX, IL-10, IL-2, CD25, Foxp3*

Abbreviations used
CMV: Cytomegalovirus
Foxp3: Forkhead box P3
IL-2R: IL-2 receptor
IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked
T_R: T-regulatory

T-regulatory (T_R) cells are critical to immune regulation and are organized into $CD25^+$ forkhead box P3 $(Foxp3)^+$ natural T_R cells and cytokine-expressing adaptive T_R cells.[1,2] Patients lacking Foxp3 have the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, characterized by diabetes mellitus, thyroiditis, eczema, severe allergies, and enteropathy.[3,4] Importantly, approximately 1/3 of patients with the IPEX syndrome do not have mutations in FOXP3.[5] Adaptive T_R cells secrete immunosuppressive cytokines (IL-10 and TGF-β), and studies suggest a role for these cells in immune regulation.[6,7] The physiologic relationship between these cell types is unknown.

CD4 lymphocytes stimulated with antibodies to the T-cell receptor subunit CD3 and the complement regulatory protein CD46 express high levels of IL-10, which is able to inhibit naive T-cell proliferation, a characteristic of adaptive T_R cells.[8] IL-2 is required for IL-10 production in this model. Previous studies have shown that activation of human CD4 lymphocytes results in the *de novo* expression of Foxp3.[9] To test whether the expression of IL-10 requires Foxp3, we studied IL-10 production by CD4 lymphocytes from 2 patients with syndromes consistent with IPEX: one patient with a known mutation in FOXP3, and a second patient with a normal FOXP3 coding sequence. These studies revealed that the Foxp3-deficient patient with IPEX expressed normal levels of IL-10, whereas the second patient exhibited defective IL-10 production because of a deficiency of CD25. Importantly, staining of CD4 lymphocytes from the CD25-deficient patient with antibodies to Foxp3 showed normal numbers of circulating $Foxp3^+$ cells. This report shows that an IPEX-like syndrome can be caused by a defect in CD25.

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Supported by National Institutes of Health grant R01-AI37618 (J.P.A.), R01-AI065617 (T.C.), the Washington University Rheumatology Postdoctoral Training Grant (A.A.C.), the Abbott Scholar Award in Rheumatology Research (J.W.V.), and the Children's Research Institute (S.T.R., J.W.V.).
Disclosure of potential conflict of interest: J. W. Verbsky has received an Abbott Scholar Award. The rest of the authors have declared that they have no conflict of interest.
Received for publication September 1, 2006; revised October 4, 2006; accepted for publication October 5, 2006.
Available online December 30, 2006.
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0091-6749/$32.00

doi:10.1016/j.jaci.2006.10.007

These studies raise important questions regarding the role of IL-2 in the function of Foxp3$^+$ cells, as well as the relationship of IL-2 and IL-10 in regulating immune responses.

METHODS

CD4 lymphocyte isolation and activation

PBMCs were obtained using Ficoll-Paque (Amersham, Piscataway, NJ) and CD4 lymphocytes isolated with magnetic beads (Miltenyi Biotec, Auburn, Calif) under an Institutional Review Board–approved human studies protocol (Washington University, St Louis, Mo). Cells were activated with plate-bound antibodies to CD3 (OKT3, American Type Tissue Collection) and CD46 (Tra2-10) with human IL-2 or human IL-15 (eBioscience, San Diego, Calif), or with 10 ng/mL phorbol 12-myristate 13-acetate and 1 μmol/L ionomycin (Sigma, St Louis, Mo).

Flow cytometry/ELISA

Lymphocytes stimulated as described for 3 days were stained with antibodies to CD69–fluorescein isothiocyanate (FN50), CD25-allophycocyanin (M-A251), CD4-phycoerythrin (RPA-T4, BD Biosciences, San Jose, Calif), and Foxp3-APC (PCH101, eBioscience) and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). Tissue culture supernatants were analyzed for IL-10 production by using an IL-10 ELISA (eBioscience).

cDNA analysis and cloning

RNA was isolated from human lymphocytes with a RNAeasy column (Qiagen, Valencia, Calif), and cDNA was generated by using the IM-PROM II reverse transcriptase system and oligo-dT (Promega, Madison, Wis). The full-length CD25 cDNA was amplified with Red-Taq DNA polymerase (Sigma) by using the following primers: 5′-ACATGGATTCATACCTGC-3′ and 5′-GATTGTTCTTCTACT CTT-3′. The 0.8-kb fragment was cloned into pCMV-Tag1 (Stratagene, La Jolla, Calif) and individual colonies sequenced. In some studies, the PCR products were concentrated and digested with BbsI (New England Biolabs, Ipswich, Mass) and separated on a 2.5% agarose gel.

Case presentation

The patient is an 8-year-old white boy born to unrelated parents with an uneventful pregnancy and delivery. At 6 weeks of age, he developed severe diarrhea, insulin-dependent diabetes mellitus, and eventual respiratory failure. Urine and blood cultures were positive for cytomegalovirus (CMV). Lung biopsy showed dense mononuclear cell lung infiltrates containing plasma cells and immunohistochemical staining positive for CMV. Intestinal biopsy showed chronic inflammation with CMV inclusion bodies. Laboratory evaluation showed a serum IgG of 850 mg/dL, IgA of 125 mg/dL, IgM of 168 mg/dL, and a lymphocyte CD3 count of 3251 cells/μL with 33% CD4 cells and 28% CD8 cells. Antiviral therapy was initiated with clearance of the CMV, but the diarrhea persisted. Repeat endoscopy at 6 months of age showed chronic inflammation with villous atrophy consistent with autoimmune enteropathy. By 2 years of age, he had developed diffuse eczema, systemic lymphadenopathy, hepatosplenomegaly, enlarged tonsils, and symptoms of sleep apnea requiring tonsillectomy and adenoidectomy. Lymph node biopsies showed lymphoid hyperplasia with evidence of EBV. By 3 years of age, he had developed hypothyroidism and severe autoimmune hemolytic anemia. CMV was again detected in his urine. Laboratory workup showed a hemoglobin of 10.7 g/dL, platelet count of 411,000/μL, white blood cell count of 10,700 cells/μL, IgA of 297 mg/dL, IgG of 1630 mg/dL, IgM of 85 mg/dL, and IgE of 120 IU/mL. At 5 years, he developed antigranulocyte antibody–positive neutropenia and had persistent sinusitis and otitis media requiring sinus surgery, repeat myringotomy tubes, and frequent antibiotics. Over the period of the next 3 years, he developed asthma, had recurrent pneumonias, and exhibited persistent lymphadenopathy, hepatosplenomegaly, eczema, and diarrhea with protein-losing enteropathy. During the course of his illness, he was treated with numerous medications, including corticosteroids, rituximab, intravenous immunoglobulin, cyclosporin, and antibiotics. Because of his enteropathy, endocrinopathy, eczema, hemolytic anemia, hepatosplenomegaly, and lymphadenopathy, he was diagnosed with an IPEX-like syndrome, and FOXP3 gene sequencing was performed, but no coding sequence mutations were found (data not shown[10]).

RESULTS

CD25 expression, but not Foxp3, is required for IL-10 production

To test whether Foxp3 expression was required for IL-10 production from human CD4 lymphocytes, 2 patients with syndromes consistent with IPEX were analyzed. One patient had a known FOXP3 coding sequence mutation (FOXP3$^-$),[3] and the second patient had a normal Foxp3 coding sequence (Foxp3$^+$, described). CD4 lymphocytes were isolated and stimulated with antibodies to CD3 and CD46, or CD3 and CD28 in the presence of IL-2. As shown in Fig 1, *A*, the Foxp3$^-$ patient expressed levels of IL-10 comparable to controls, whereas the Foxp3$^+$ patient failed to express detectable IL-10. Because this model of IL-10 production is dependent on exogenous IL-2, and because animal models deficient in the IL-2 receptor subunits have autoimmune phenomena similar to mice deficient in Foxp3, we examined the IL-2 receptor components in the Foxp3$^+$ patient.[8,11,12] Activation of CD4 lymphocytes from a control subject resulted in strong upregulation of CD25, whereas no CD25 was detected on CD4 lymphocytes from the Foxp3$^+$ patient with IPEX (Fig 1, *B*). The early activation marker CD69, however, was induced in this patient's lymphocytes. Pharmacologic activation with phorbol 12-myristate 13-acetate and ionomycin resulted in upregulation of CD25 and CD69 from normal lymphocytes, but only CD69 was expressed in the Foxp3$^+$ patient (data not shown).

Because no CD25 was expressed from the Foxp3$^+$ patient, sequence analysis was performed on the CD25 gene from PCR-amplified cDNA. Sequence analysis revealed a single base pair insertion after position 692 in 1 allele of his CD25 gene that resulted in a frameshift mutation just proximal to the transmembrane domain (Fig 1, *C*). Transfection studies showed that this protein was not surface-expressed and did not prevent the expression of wild-type CD25 (data not shown). Sequence analysis of the patient's mother confirmed the single base pair insertion in one of her CD25 alleles (data not shown). Genomic sequencing revealed a second allele with a C to T substitution at position 301 resulting in a stop codon (Fig 1, *C*). Sequence analysis of the patient's father confirmed the single base pair substitution at position 301 (data not shown). Finally, PBMCs were isolated from

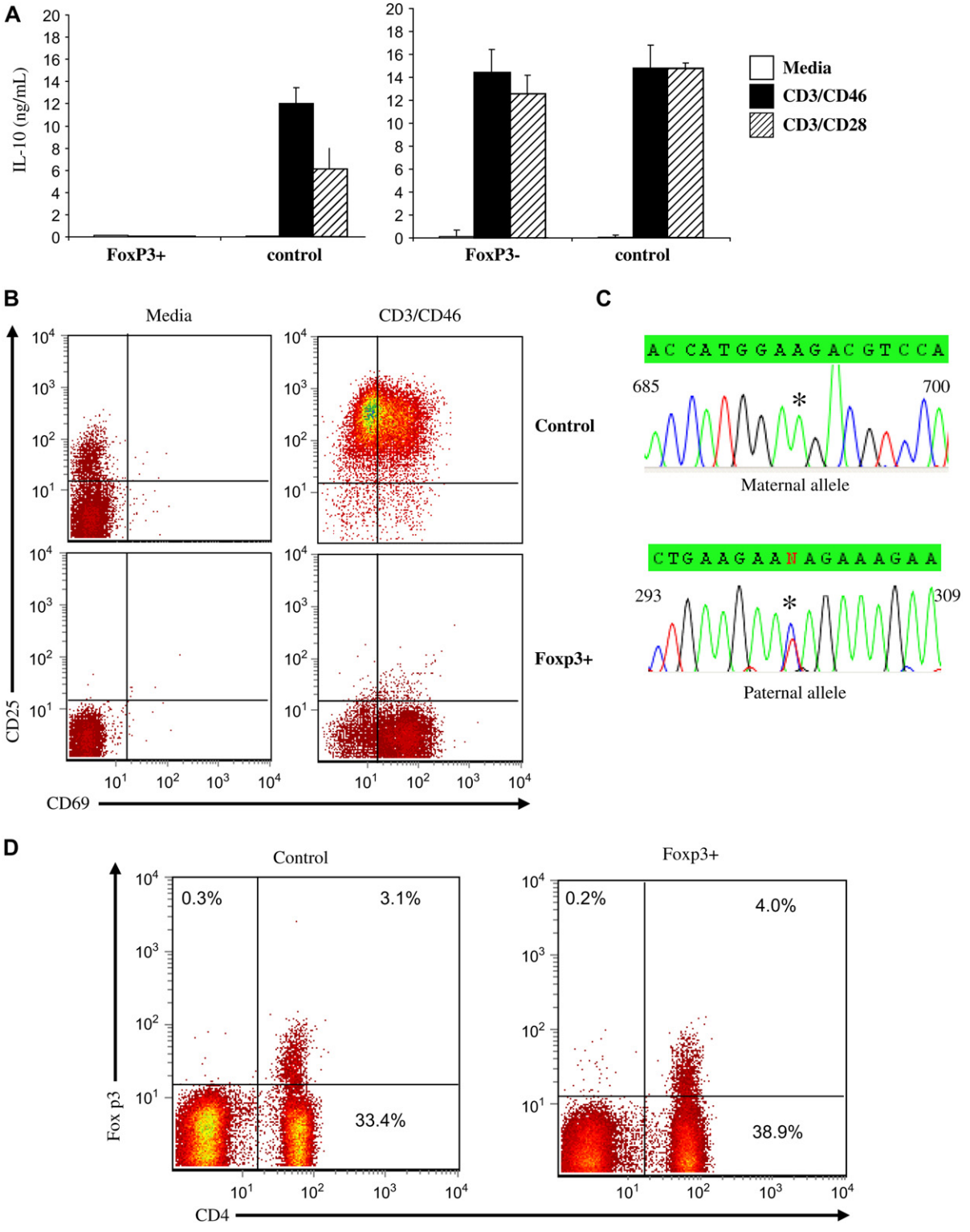

FIG 1. CD25 deficiency results in defective IL-10 expression from CD4 cells, but is not required for Foxp3$^+$ T_R generation. **A,** CD4 lymphocytes from a Foxp3 mutant (Foxp3$^-$) and a Foxp3 normal (Foxp3$^+$) patient were activated with IL-2 (10 U/mL) for 3 days, and IL-10 was assayed by ELISA. **B,** CD4 lymphocytes from control and the Foxp3$^+$ patient were activated for 3 days and stained with antibodies to CD25 and CD69. **C,** Sequence trace of maternal allele showing single adenine insertion *(*)* after position 692 *(top)*. Sequence trace of genomic DNA showing C to T substitution at position 301 (*) of paternal allele *(bottom)*. **D,** PBMCs from the Foxp3$^+$ patient were isolated and stained with antibodies to CD4 and Foxp3.

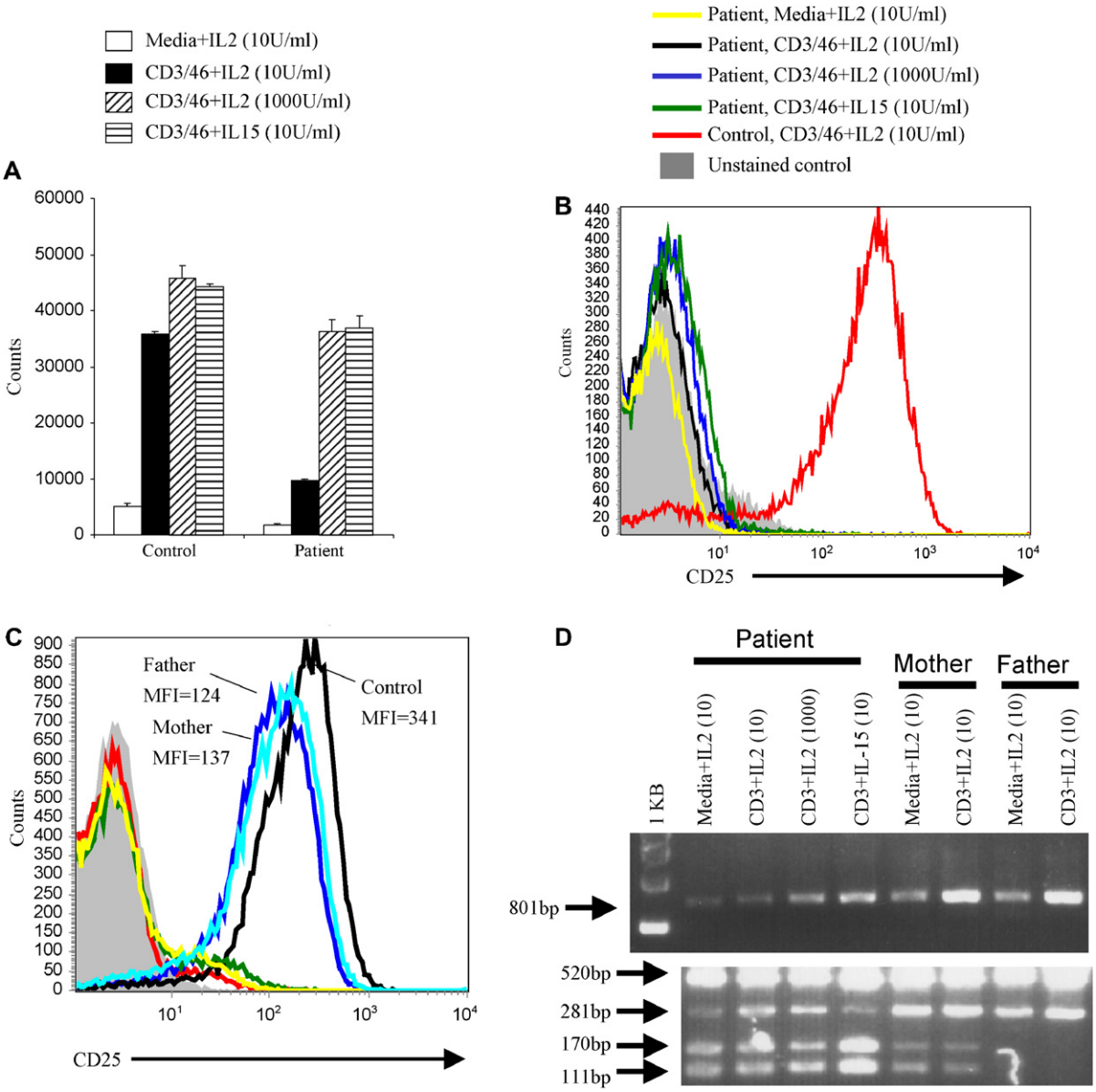

FIG 2. IL-15 and high-dose IL-2 rescue the proliferative defect of a CD25-deficient patient. CD4 lymphocytes were activated for 3 days and **(A)** proliferation measured by ^{3}H-thymidine incorporation and **(B)** CD25 expression measured by flow cytometry. **C,** CD4 lymphocytes were isolated and stimulated as shown, and CD25 expression was measured 3 days later. **D,** CD4 lymphocytes were activated for 3 days, and RT-PCR products of the CD25 gene were concentrated and digested with BbsI. *MFI,* Mean fluorescence intensity; *1 KB,* 1 KB molecular weight marker.

the Foxp3^{+} patient and stained with a Foxp3 antibody. As shown in Fig 1, *D,* a similar percentage of CD4 lymphocytes from this patient expressed Foxp3 compared with a normal control. No cells from this patient expressed CD25 (data not shown).

IL-15 and high-dose IL-2 rescued proliferation but not CD25 expression

To confirm that CD25 deficiency accounts for the phenotype of this patient, IL-15 was used to rescue the CD25 defect. IL-2 and IL-15 each use the IL-2Rβ and IL-2Rγ subunits for signal transduction, only differing in the IL-2Rα (CD25) and IL-15 receptor α subunits to determine ligand specificity and sensitivity.[13] High doses of IL-2 can signal without CD25, using the β-chain and γ-chain of the IL-2 receptor only. As shown in Fig 2, *A,* culture of the patient's CD4 lymphocytes on plate-bound CD3 and CD46 with a low dose of IL-2 (10 U/mL) resulted in poor proliferation compared with a normal volunteer. Culture with high-dose IL-2 (1000 U/mL) or IL-15 (10 U/mL) resulted in normalization of the proliferative defect, confirming that the rest of the downstream signaling

pathway is intact in this patient. We next tested whether this restored the expression of CD25. As shown in Fig 2, *B,* activation of CD4 lymphocytes from a normal donor resulted in CD25 expression, whereas activation of the patient's CD4 lymphocytes in the presence of high-dose IL-2 or IL-15 resulted in no CD25 expression compared with normal controls. In addition, high-dose IL-2 (1000 U/mL) and IL-15 (10 U/mL), but not low-dose IL-2 (10 U/mL), were able to induce measurable levels of IL-10 from this patient's CD4 cells, although the response was reduced in comparison with normal controls (data not shown).

Parental analysis suggests that each carries distinct nonfunctional alleles

Because CD25 is an autosomal gene, both the mother and father should express roughly 50% of the amount of CD25 on activated lymphocytes if each has a defective allele. Indeed, CD25 staining of activated lymphocytes from both parents showed approximately half the mean fluorescence intensity compared with normal controls (Fig 2, *C*). Furthermore, CD4 cells stimulated as in Fig 2, *C,* were analyzed by RT-PCR for CD25 expression. As shown in Fig 2, *D,* stimulation with CD3/CD46 in the presence of IL-15 or high-dose IL-2 resulted in transcriptional upregulation of CD25. Because the single base pair insertion of the CD25 allele results in a novel BbsI restriction site, BbsI digestion of PCR products resulted in 520-bp and 281-bp products in one allele, and 520-bp, 170-bp, and 111-bp products in the second allele. These results show the presence of the BbsI restriction site in both the patient and his mother, but not in the patient's father. In addition, the majority of the induced transcript in this patient was the maternal allele because the ratio of the PCR signal of maternal to paternal allele increased on activation compared with the mother's cDNA (Fig 2, *D*). This implies that the father's allele was not efficiently upregulated on activation, likely because the stop codon resulted in nonsense-mediated mRNA decay.[14]

DISCUSSION

Herein we describe a patient with an IPEX-like syndrome with a normal Foxp3 gene but with a deficiency in CD25. He carries a single base pair insertion of one allele of his CD25 gene and a second allele with a substitution resulting in a stop codon. Analysis of CD25 expression from parental lymphocytes shows approximately half the amount of CD25 on the surface during T-cell activation, supporting the notion that each is heterozygous at the CD25 locus. Importantly, the proliferative defect of this patient's CD4 cells can be rescued with IL-15 or high concentrations of IL-2, but under these conditions, CD25 is not expressed. Finally, Foxp3 analysis of PBMCs from this patient show a relatively normal percentage of $Foxp3^+$ cells, but IL-10 expression from CD4 cells was defective.

These data argue that IL-2 responsiveness is important for IL-10 production from CD4 cells. We have confirmed these findings with CD4 lymphocytes from CD25-deficient mice, which also showed defective IL-10 expression (data not shown). Both adaptive T_R cells and natural T_R cells have been reported to express IL-10, although the *in vivo* significance of IL-10 production by these cell types is unclear.[2] This patient exhibited features distinct from Foxp3-deficient patients with IPEX, including intense inflammatory responses to CMV resulting in respiratory failure, and enteropathy despite a lack of food allergies or a significantly elevated IgE level. This patient lacked IL-10 production from adaptive T_R cells with low doses of IL-2, whereas a Foxp3-deficient patient did not. Thus, IL-10 produced by adaptive T_R cells in response to IL-2 may dampen immune responses at sites of persistent microbial challenge or at sites of inflammation. Furthermore, the CD25-deficient patient expressed normal numbers of circulating $Foxp3^+$ cells, implying that CD25 is required for the function but not the survival of natural T_R cells in human beings. This contrasts with mouse models in which CD25 is required for the survival of $Foxp3^+$ cells, but their suppressive functions were reported to be normal.[15] IL-2 may therefore be required for the production of immunosuppressive cytokines (ie, IL-10, TGF-β) from natural T_R cells, or may prime natural T_R cells for other suppressive abilities. Finally, the patient described in this report had persistent EBV and CMV infections, as well as frequent episodes of pneumonia and sinusitis. These observations argue that IL-2 has both stimulatory and regulatory properties in human beings.

The fact that this patient exhibited a majority of the clinical features of the IPEX syndrome, including endocrinopathies, eczema, hemolytic anemia, lymphadenopathy, hepatosplenomegaly, and enteropathy, argues that the IPEX syndrome should be broadened or renamed to include CD25 deficiency. Studies in mice support these findings, because CD25-deficient mice exhibit a similar phenotype compared with Foxp3-deficient animals.[16] An additional patient with CD25 deficiency was described by Roifman[17] who exhibited many of the same features described in this patient, including chronic viral infections, lymphadenopathy, and lymphocytic inflammation of lung, liver, and gut. Roifman's patient, however, did not have evidence of diabetes or other endocrinopathies that typically characterize IPEX. Thus, CD25 deficiency differs from Foxp3 deficiency by an increased susceptibility to viral infections and variable penetrance of endocrinopathies. What proportion of patients with syndromes consistent with IPEX but with normal Foxp3 gene results from CD25 deficiency is unknown, but defects in IL-2 or its receptor subunits should be considered in any patient with symptoms reminiscent of IPEX in early childhood.

We thank Dr William Grossman and Dr Calvin Williams for critical review of the manuscript. We also thank Karen Siegrist for assisting in sample procurement.

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