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Kristin A. Lambert, BS^a
Man-Hsun Hsu, BS^a
Zoe C. Laryea, BS^a
Yuanjun Zhao, MD, PhD^b
Alanna N. Roff, MS^c
Jeffrey M. Sundstrom, MD, PhD^b
Faoud T. Ishmael, MD, PhD^{a,d}

From ^athe Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, and ^bthe Department of Ophthalmology, Penn State Milton S. Hershey Medical Center, Hershey, Pa; ^cthe Department of Biostatistics and Informatics, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colo; and ^dthe Department of Allergy and Sleep Medicine, Mount Nittany Medical Group, State College, Pa. E-mail: faoud.ishmael@mountnittany.org.

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REFERENCES

- Calderon MA, Linneberg A, Kleine-Tebbe J, De Blay F, Hernandez Fernandez de Rojas D, Virchow JC, et al. Respiratory allergy caused by house dust mites: what do we really know? *J Allergy Clin Immunol* 2015;136:38-48.
- Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol* 2011;32:402-11.
- Kulshreshtha A, Ahmad T, Agrawal A, Ghosh B. Proinflammatory role of epithelial cell-derived exosomes in allergic airway inflammation. *J Allergy Clin Immunol* 2013;131:1194-203.e1-14.
- Admyre C, Bohle B, Johansson SM, Focke-Tejkl M, Valenta R, Scheynius A, et al. B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines. *J Allergy Clin Immunol* 2007;120:1418-24.
- Prado N, Marazuela EG, Segura E, Fernandez-Garcia H, Villalba M, Thery C, et al. Exosomes from bronchoalveolar fluid of tolerized mice prevent allergic reaction. *J Immunol* 2008;181:1519-25.
- Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci* 2018;75:193-208.
- Gon Y, Maruoka S, Inoue T, Kuroda K, Yamagishi K, Kozu Y, et al. Selective release of miRNAs via extracellular vesicles is associated with house-dust mite allergen-induced airway inflammation. *Clin Exp Allergy* 2017;47:1586-98.
- Nocera AL, Mueller SK, Stehan JR, Hing L, Seifert P, Han X, et al. Exosome swarms eliminate airway pathogens and provide passive epithelial immunoprotection through nitric oxide. *J Allergy Clin Immunol* 2019;143:1525-35.e1.

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The FOXP3Δ2 isoform supports Treg cell development and protects against severe IPEX syndrome



To the Editor:

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare syndrome of immune dysregulation characterized by severe autoimmunity that is often fatal in early life. IPEX syndrome is caused by mutations in the forkhead box protein 3 (*FOXP3*) gene on chromosome Xp11.23.¹ *FOXP3* is a key transcription factor for the development and function of regulatory T (Treg) cells, which are essential for immune tolerance.¹

FOXP3 protein is comprised of 4 functional domains with distinct roles in gene regulation: a carboxyl terminal forkhead domain important for nuclear localization and DNA binding and 3 protein/protein interaction domains, including a central zinc finger, a leucine zipper, and a proline-rich transcriptional repressor domain (see Fig E1 in this article's Online Repository at www.jacionline.org).¹ Alternative splicing of human *FOXP3* results in expression of 4 naturally occurring isoforms: a full-length protein containing all domains (*FOXP3fl*) and shortened isoforms as a result of exclusion of either exon 2 (*FOXP3Δ2*), exon 7 (*FOXP3Δ7*), or both exons 2 and 7 (*FOXP3Δ2Δ7*) from the *FOXP3* transcripts.²⁻⁴ Differential expression of *FOXP3* isoforms has been found in patients with diseases, such as Crohn disease, although the regulation and function of distinct isoforms are incompletely understood.⁵ This is compounded by the fact that mice naturally express only the full-length protein, thus limiting the utility of mouse models to elucidate the functional significance of distinct *FOXP3* isoforms in human subjects.²⁻⁴ The most abundant isoforms of human *FOXP3* are *FOXP3fl* and *FOXP3Δ2*.^{1,4} Recently, we have found that *FOXP3Δ2* is preferentially induced on Treg cell activation and that high *FOXP3Δ2* use is associated with atherosclerotic plaque stability.⁴ However, to date, it has been impossible to study the *in vivo* function of either *FOXP3* isoform. Exceedingly rare reports of IPEX syndrome caused by mutations predicted to cause loss of exons 2 or 7, which have assessed isoform expression, have not provided specific clinical data or detailed Treg cell analysis.^{1,5-7}

Here we report the molecular phenotype of 2 patients with an autoimmune phenotype and a carrier female subject from the same family, with a novel *FOXP3* frameshift mutation (c.305delT; p.Phe102fs), which results in loss of *FOXP3fl* and *FOXP3Δ7* expression. The proband (P1) is a 25-year-old man who had type 1 diabetes (insulin-dependent diabetes mellitus [IDDM]) at 6 months of age and was strongly positive for glutamic acid decarboxylase autoantibodies. In adolescence he had inflammatory bowel disease and primary sclerosing cholangitis, both of which have gone into long-term remission with minimal intervention. He possessed additional autoantibodies, including anti-PR3, anti-gliadin IgA/IgG, as well as ASCA, and had normal lymphocyte subsets and levels of serum immunoglobulins (see Table E1 in this article's Online Repository at www.jacionline.org). Since late adolescence, he has had recurrent staphylococcal boils, sometimes requiring drainage. He has one sister who is well and not a carrier.

P2 is the 72-year-old maternal grandfather of P1, who was in good health until 40 years of age when he had psoriatic arthritis. He has type 2 diabetes and chronic obstructive pulmonary disease, having smoked heavily until 5 years prior. There is no other family history of note (see Fig E2 in this article's Online Repository at www.jacionline.org).

Panel next-generation sequencing testing (Agilent custom capture v3 Illumina/HiSeq; Illumina, San Diego, Calif) in P1 at age 20 years identified a novel hemizygous frameshift mutation in exon 2 of *FOXP3* (NM_014009.3:c.305delT), which is suggestive of IPEX syndrome (see Methods in this article's Online Repository at www.jacionline.org). *FOXP3* exon 2 encodes amino acids 71 to 105 of the 431-amino-acid protein, and c.305delT is predicted to result in protein termination at amino acid 102 (p.Phe102fs). Familial segregation studies using Sanger sequencing confirmed the patient's mother (C1), aged 47 years and healthy, to be a carrier of this variant. c.305delT was also

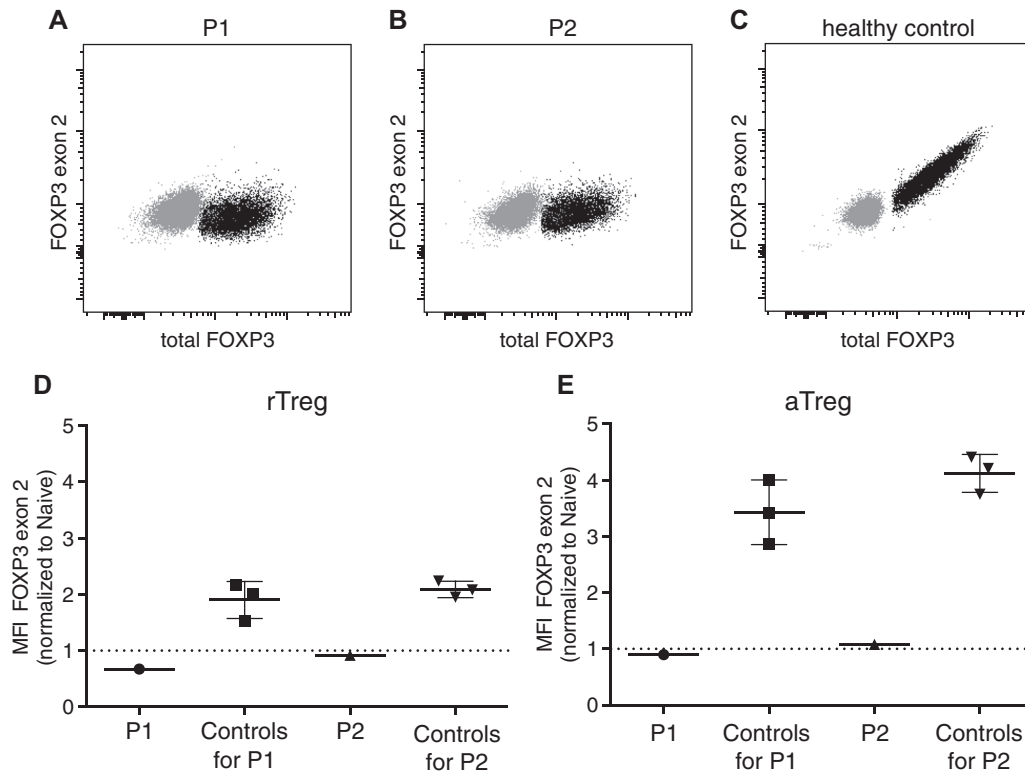


FIG 1. Altered FOXP3 isoform expression caused by the c.305delT mutation. **A–C**, FACS staining with anti-FOXP3 antibody targeting the exon 2–encoded domain (clone 150D/E4, *y*-axis) versus anti-FOXP3 antibody targeting an epitope common to all FOXP3 isoforms (clone 236A/E7, *x*-axis). **Black plots** are gated on $CD4^+CD25^+CD127^{lo}$ FOXP3total cells and show the signal from the FOXP3 exon 2 antibody (*y*-axis) is absent in FOXP3⁺ cells from the proband P1 (Fig 1, **A**) and his grandfather P2 (Fig 1, **B**) in contrast to a representative healthy control subject age and sex matched to the proband (Fig 1, **C**). **D** and **E**, Mean fluorescence intensity (MFI) of FOXP3 containing exon 2 signal relative to naive T cells (gray plots; $CD4^+CD45RA^+FOXP3^+$) in resting Treg cells (rTreg; $CD4^+CD25^+CD127^{lo}$ TotalFOXP3⁺; Fig 2, **D**) or aTreg cells ($CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ TotalFOXP3⁺; Fig 2, **E**) and compared with age- and sex-matched healthy control subjects.

identified in P2, establishing a new diagnosis of IPEX syndrome at 70 years of age (see Fig E3 in this article's Online Repository at www.jacionline.org). Importantly, there were no alterations in alternative splicing of *FOXP3* in the patients (see Fig E4 in this article's Online Repository at www.jacionline.org).

Both P1 and P2 express FOXP3 protein (mAb “FOXP3total” clone 236A/E7; targeting a sequence encoded by exons 3–6, which is common to all FOXP3 isoforms) but lacked the exon 2–coded region of the proline-rich repressor domain (mAb “FOXP3ex2” clone 150D/E4, targeting FOXP3 exon 2–coded sequence), confirming lack of isoforms containing exon 2, FOXP3fl, and FOXP3Δ7 (Fig 1, **A–C**). Both activated Treg (aTreg) cells ($CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ TotalFOXP3⁺) and resting Treg cells ($CD4^+CD25^+CD127^{lo}$ TotalFOXP3⁺), as previously defined, in P1 and P2 lacked expression of FOXP3 isoforms containing exon 2 (Fig 1, **D** and **E**).⁸ Of note, P2 presents with an increased proportion of Treg cells, mainly because of an increase in aTreg cell proportion, possibly as a result of a compensatory mechanism to overcome altered function caused by loss of FOXP3fl expression (Fig 2, **A–D**). Additional analysis of $CD4^+$ and $CD8^+$ effector T cells revealed that P1 presents a decreased proportion in naive effector T cells, with marked reductions in proportions of $CD45RA^+CCR7^+$ cells (see Fig E5, **A** and **B**, in this article's Online Repository at www.jacionline.org).

We further established that contraction of the naive $CD4^+$ T-cell population resulted in a relative increase in numbers of central memory cells, whereas the paucity of naive $CD8^+$ T cells coincided with an expansion of $CD45RA^+$ effector memory cells (see Fig E5, **A** and **B**). $CD4^+$ T cells from both P1 and P2 showed a greater degree of activation, as evidenced by a greater proportion of Ki67⁺ cells, compared with control $CD4^+$ T cells (see Fig E5, **C**).

Because of random inactivation of the X-chromosome, the healthy carrier mother had 2 distinct populations of Treg cells, one expressing the wild-type *FOXP3* gene and leading to normal isoform expression pattern and a smaller population of Treg cells (17% of the total Treg cell pool) expressing the c.305delT mutation with loss of FOXP3 exon 2 signal (Fig 2, **E**). This suggests a loss of fitness in Treg cells lacking FOXP3fl.

We demonstrate the presence of naturally occurring Treg cells expressing FOXP3Δ2 in the absence of FOXP3fl in 2 patients and a carrier female subject caused by a novel *FOXP3* mutation in exon 2 (c.305delT). We confirm that in the absence of exon 2, FOXP3Δ2 is expressed and can support development of the aTreg cell phenotype ($CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ TotalFOXP3⁺), which supports previous *in vitro* findings.⁹ Finally, our data reveal that FOXP3fl contributes to the maintenance of Treg cells.

IPEX syndrome has been reported in 4 patients with frameshift mutations predicted to affect exon 2.^{1,6,7} These include

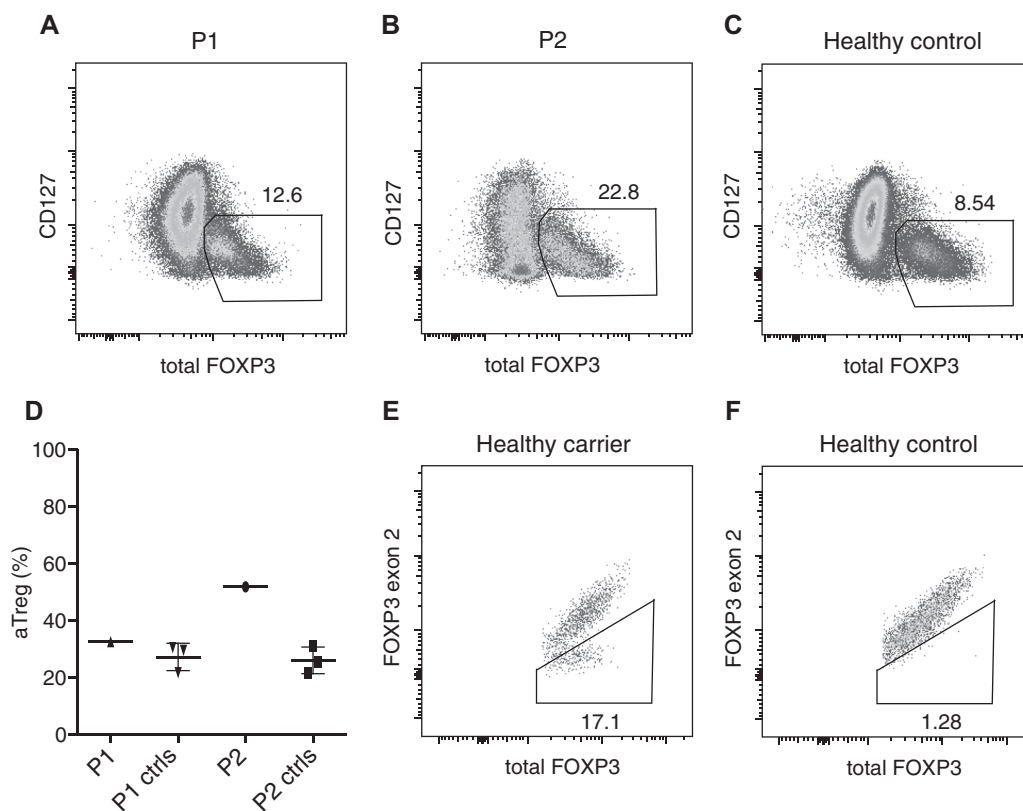


FIG 2. Peripheral blood of P2 presents an unusually high proportion of activated FOXP3⁺CD4⁺ T cells. **A-C**, Flow cytometric analysis of CD127 and FOXP3 expression in CD4⁺ T cells in the proband P1 (Fig 2, A), grandfather P2 (Fig 2, B), and a representative healthy control subject age matched to the proband (Fig 2, C). **D**, Quantification of aTreg cells (aTreg; CD4⁺CD25^{hi}CD127^{lo}CD45RA⁺ TotalFOXP3⁺) of all Treg cells in P1 and P2 in comparison with age- and sex-matched healthy control subjects. **E** and **F**, Proportion of FOXP3 exon 2-positive and negative Treg cells (CD4⁺CD25⁺CD127^{lo}FOXP3) in the carrier mother (Fig 2, E) in comparison with an age- and sex-matched representative control subject (Fig 2, F).

(1) 2 recently published patients with deletions in exon 2 (c.232_233del and c.239del) with no clinical history provided; (2) a 6-month-old infant with failure to thrive, IDDM, glomerulonephritis, autoimmune hemolytic anemia, eczema, chronic diarrhea, and hypothyroidism, who had a 2-bp deletion (c.303-304delTT) and underwent hematopoietic stem cell transplantation in early childhood; and (3) an adolescent with infant-onset enteropathy, autoimmune hemolytic anemia, autoimmune thyroiditis, and renal tubular dysfunction who had a 1-bp deletion (c.227delT) and has been managed with immunosuppression (see Table E2 in this article's Online Repository at www.jacionline.org).^{1,6,7} The attenuated phenotype seen in the latter patient and in P1 and P2 detailed here is the first evidence that FOXP3Δ2 alone can confer clinically relevant suppressive activity in Treg cells.

Protein studies in patients with the c.232_233del and c.239del mutations demonstrated an absence of FOXP3fl and reduced proportion of Treg cells, as defined by CD25⁺FOXP3⁺ expression; however, individual patients' Treg cell analyses were not published.¹ Studies in the c.227delT patient did not examine isoform expression and found a lower proportion of Treg cells compared with those in healthy control subjects but, in accordance with our findings, demonstrated a high percentage of activated (ie, CD45RO⁺) T cells.⁷ The authors hypothesized that the high proportion of activated T cells compared with

disease control subjects might be due to the severe phenotype of the disease, but in our patients a high proportion of aTreg cells was associated with a milder phenotype, particularly in P2. The degree of activation does not appear to be an age-related phenomenon because both patients had greater proportions of aTreg cells compared with age-matched control subjects; however, these distinct phenotypes can be explained by a difference in Treg cell function.

To date, no studies of Treg cell function with exon 2 mutations have been published. *In vitro* studies using ectopic expression of FOXP3Δ2 have shown that this isoform can induce an aTreg cell phenotype with dysregulated expression of specific genes and some suppressive function.^{2,3,9} Both P1 and P2 demonstrated evidence of cellular immune dysregulation, with a reduction in numbers of naive CD4⁺ and CD8⁺ T cells and increased numbers of CD4⁺ central memory, CD8⁺ effector, and CD45RA⁺ revertant memory T cells in P1 (see Fig E5). However, the milder clinical phenotype described here strongly supports a functional role for FOXP3Δ2, and the strikingly mild case of P2 possibly results in part from a greater compensatory increase in Treg cell numbers caused by epigenetic and environmental factors.

In the carrier state we observed 2 populations of Treg cells, with a bias toward Treg cells expressing wild-type FOXP3 (Fig 2, E). This finding suggests that the higher number of Treg cells observed in P2 was compensatory rather than caused by an

increased proliferative capacity of cells expressing the exon 2 mutation. Our finding of bimodal FOXP3 expression in the carrier differs from the only other published report of a carrier with an exon 2 mutation (c.227delT) who expressed only one population of CD4⁺CD25⁺CD127^{lo} cells, all expressing WT FOXP3.⁷ Our results are the first to confirm the natural ability of isoforms lacking exon 2 to promote their own transcription, resulting in expression of a functional isoform of FOXP3 that can support Treg cell development.

In conclusion, we report a family with autoimmunity across 3 generations, including 2 affected male subjects. We have shown that a milder IPEX phenotype was due to selective deletion of FOXP3 exon 2 expression, resulting in loss of FOXP3fl but retained expression of FOXP3Δ2. This report confirms that FOXP3Δ2 can support Treg cell development *in vivo* and mitigate at least some of the clinical features of complete FOXP3 deficiency, as observed in patients with classic IPEX syndrome. These findings provide powerful patient-derived evidence for the functional capabilities of the FOXP3Δ2 isoform. The variable penetrance is important because it suggests that future patients might be identified in populations with milder autoimmunity not reaching the criteria for IPEX syndrome.

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Katie Frith, MD^{a,b}
Anne-Laure Joly, PhD^c
Cindy S. Ma, PhD^{d,e}
Stuart G. Tangye, PhD^{d,e}
Zuzana Lohse, PhD^f
Christina Seitz, PhD^c
Charles F. Verge, PhD^{b,g}
John Andersson, PhD^c
Paul Gray, MD^{a,b}

From ^athe Department of Immunology and Infectious Diseases and ^bthe Department of Endocrinology, Sydney Children's Hospital, Sydney, Australia; ^cthe School of Women's and Children's Health and ^dSt Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Sydney, Australia; ^ethe Immunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden; ^fthe Immunology Division, Garvan Institute of Medical Research, Darlinghurst, Australia; and ^gthe Department of Clinical Genetics, Vejle Hospital, Vejle, Denmark. E-mail: Catherine.frith@health.nsw.gov.au.

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REFERENCES

- Gambineri E, Ciullini Mannurita S, Hagin D, Vignoli M, Anover-Sombke S, DeBoer S, et al. Clinical, immunological, and molecular heterogeneity of 173 patients with the phenotype of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. *Front Immunol* 2018;9:2411.
- Allan SE, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, et al. The role of 2 FOXP3 isoforms in the generation of human CD4⁺ Tregs. *J Clin Invest* 2005;115:3276-84.
- Smith EL, Finney HM, Nesbitt AM, Ramsdell F, Robinson MK. Splice variants of human FOXP3 are functional inhibitors of human CD4⁺ T-cell activation. *Immunology* 2006;119:203-11.
- Joly AL, Seitz C, Liu S, Kuznetsov NV, Gertow K, Westerberg LS, et al. Alternative splicing of FOXP3 controls regulatory T cell effector functions and

is associated with human atherosclerotic plaque stability. *Circ Res* 2018;122:1385-94.

- Harbuz R, Lespinasse J, Boulet S, Francannet C, Creveaux I, Benkhelifa M, et al. Identification of new FOXP3 mutations and prenatal diagnosis of IPEX syndrome. *Prenat Diagn* 2010;30:1072-8.
- Moudgil A, Perriello P, Loechelt B, Przygodzki R, Fitzgerald W, Kamani N. Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome: an unusual cause of proteinuria in infancy. *Pediatr Nephrol* 2007;22:1799-802.
- Otsubo K, Kanegane H, Kamachi Y, Kobayashi I, Tsuge I, Imaizumi M, et al. Identification of FOXP3-negative regulatory T-like (CD4(+)CD25(+)CD127(low)) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *Clin Immunol* 2011;141:111-20.
- Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity* 2009;30:899-911.
- Aarts-Riemens T, Emmelot ME, Verdonck LF, Mutis T. Forced overexpression of either of the two common human Foxp3 isoforms can induce regulatory T cells from CD4(+)CD25(-) cells. *Eur J Immunol* 2008;38:1381-90.

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Distribution of subsets of blood monocytic cells throughout life



To the Editor:

Currently, it is well established that monocytes are a heterogeneous type of cell consisting of phenotypically and functionally distinct subpopulations found to be numerically altered in blood in patients with a wide variety of disease conditions, such as infection, autoimmunity, respiratory and cardiovascular diseases, and inflammatory disorders.^{1,2} Thus, 3 subpopulations of circulating monocytic cells have been identified based on expression of the CD14 LPS receptor and the CD16 low-affinity Fc IgG receptor: (1) CD14^{hi}CD16⁻ classical monocytes (cMos), (2) CD14^{hi}CD16⁺ intermediate monocytes (iMos), and (3) CD14^{-lo}CD16⁺ nonclassical monocytes (ncMos).³ Monocytes circulate in blood for up to 3 days until recruited to virtually any human tissue, where they differentiate into either tissue macrophages or myeloid dendritic cells.⁴ Then, tissue macrophages can migrate from their tissue location through the lymph system⁵ before they potentially die outside the circulation.⁶

Despite our knowledge of the biology of monocytes increasing in recent years, normal reference ranges for the distinct monocyte subsets in blood throughout life (eg, from cord blood [CB] and newborns to elderly subjects) have never been systematically defined. Moreover, the precise maturational and functional relationship between the distinct populations of blood monocytes and their tissue distribution profiles remains unknown.

To provide a frame of reference for future identification of disease-associated altered profiles, we investigated the distribution of distinct monocytic cell subsets in normal blood versus secondary lymphoid tissues, such as bone marrow (BM), lymph node, and spleen. Our aim was to shed light on changes in the distribution of these monocytic cell subsets in blood and other lymphoid tissues. For this purpose, a total of 188 EDTA-anticoagulated blood samples were studied: 11 CB specimens from full-term neonates and 177 blood samples from 164 healthy subjects and 13 solid organ donors (102 male and 75 female

METHODS

Extended clinical history

The proband P1 is a 24-year-old Australian man with type 1 diabetes mellitus (IDDM), inflammatory bowel disease, and primary sclerosing cholangitis. He was given a diagnosis of type 1 diabetes mellitus at 6 months of age. Other than IDDM, he remained well until adolescence, when he experienced a slowing of growth, abdominal pain, iron deficiency anemia, and delayed puberty. Endoscopy revealed villous atrophy, and subsequently, at 18 years of age, a diagnosis of inflammatory bowel disease was made with diffuse active colitis, crypt abscesses, and occasional granulomas noted on colonic biopsy extending up to the terminal ileum and responsive to mesalazine. At this time, he was also given a diagnosis of primary sclerosing cholangitis based on his cholestatic pattern of deranged liver function tests associated with hypergammaglobulinemia (IgG, 20.6 g/L) and positive autoantibodies, including anti-neutrophil cytoplasmic antibodies, with increased levels of proteinase 3 antibody (15 EU [0-5 EU]). Other autoantibodies included increased gliadin IgA and IgG levels (21 and 19 U, respectively; normal range, 0-7 U). He had increased *Saccharomyces cerevisiae* IgA and IgG levels (63 and 70 U/mL, respectively; normal range, 0-19 U/mL), which are known to be associated with colitis. P1 had a negative anti-nuclear antibody result, tissue transglutaminase IgA, endomysial IgA, smooth muscle, mitochondria, gastric parietal cell, and liver kidney microsomal antibodies. A liver biopsy was not performed, and he responded well to ursodeoxycholic acid. Since late adolescence, he has had a number of infectious presentations, including recurrent staphylococcal cutaneous boils requiring incision and drainage and aseptic meningitis attributed to a drug reaction to Bactrim (sulfamethoxazole and trimethoprim).

P2 is the Danish grandfather of P1 and was in good health until 40 years of age, when he had moderate-to-severe psoriatic arthritis. He has type 2 diabetes and chronic obstructive lung disease, having smoked heavily until 5 years prior. He was one of 4 children, all male, born to the same father but with different mothers (see pedigree). One of his paternal half-brothers died from pneumonia at 5 years of age. There is no other family history of note.

Gene analysis

Coding regions of a panel of neonatal diabetes genes were sequenced by using the Agilent custom capture v3 Illumina/HiSeq. Sanger sequencing was used to confirm variants and for family segregation studies.

Flow cytometry

Fluorescence-activated cell sorting (FACS) analyses were performed on frozen PBMCs for the carrier. PBMCs were separated by using Ficoll-Hypaque density gradient separation and resuspended in cryoprotective medium (90% FCS and 10% dimethyl sulfoxide) and then frozen by using

controlled rate freezing and stored long term in vapor phase LN2. For P1 and P2, PBMCs were separated by means of Ficoll gradient centrifugation (GE Healthcare, Chicago, Ill), and fresh cells were used for FACS analysis. PBMCs were stained with the Live/Dead fixable stain kit (Life Technologies, Grand Island, NY), followed by surface marker staining against CD3 (HIT3a; BioLegend, San Diego, Calif), CD4 (RPA-T4 and SK3 pBD; BD Biosciences, San Jose, Calif), CD8 (RPA-T8; BD), CD25 (CD25-3G10; Invitrogen, Carlsbad, Calif), CD127 (eBioRDR5; eBioscience, San Diego, Calif), CCR7 (G042H7; BioLegend), and CD45RA (HI100, BioLegend). Cells were then fixed and permeabilized according to the protocol by using the FOXP3 staining kit (eBioscience) and stained for Ki67 (B56; BD Biosciences), as well as FOXP3 isoforms, by using anti-FOXP3total (clone 236A/E7, targeting a sequence encoded by exon 3-6, common to all FOXP3 isoforms) and anti-FOXP3ex2 (clone 150D/E4, targeting FOXP3 exon 2-coded sequence; both from eBioscience). Cell signals were acquired on an FACS Fortessa (BD Biosciences) and analyzed with FlowJo software (version 7.6.4; TreeStar, Ashland, Ore). Treg cells were defined as CD4⁺CD127^{low}CD25⁺FOXP3⁺ cells, as previously described.^{E1}

X-inactivation methods

The microsatellite associated with the promoter of the androgen receptor gene (AR locus, also known as HUMARA locus-Xq12) was amplified by using PCR with FAM-labeled primers from uncut genomic DNA and from genomic DNA digested with methylation-sensitive restriction enzymes (*HhaI* and *HpaII*). PCR fragments were resolved by means of denaturing gel electrophoresis on an ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, Calif). Fragment separation was performed at the AGRF Westmead laboratory. Nonskewed X-inactivation is interpreted because the most active allele is less than 75% active.

REFERENCES

- E1. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity* 2009;30:899-911.
- E2. Otsubo K, Kanegane H, Kamachi Y, Kobayashi I, Tsuge I, Imaizumi M, et al. Identification of FOXP3-negative regulatory T-like (CD4(+)CD25(+)CD127(low)) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *Clin Immunol* 2011;141:111-20.
- E3. Moudgil A, Perriello P, Loechelt B, Przygodzki R, Fitzgerald W, Kamani N. Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome: an unusual cause of proteinuria in infancy. *Pediatr Nephrol* 2007;22:1799-802.
- E4. Gambineri E, Ciullini Mannurita S, Hagin D, Vignoli M, Anover-Sombke S, De-Boer S, et al. Clinical, immunological, and molecular heterogeneity of 173 patients with the phenotype of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. *Front Immunol* 2018;9:2411.

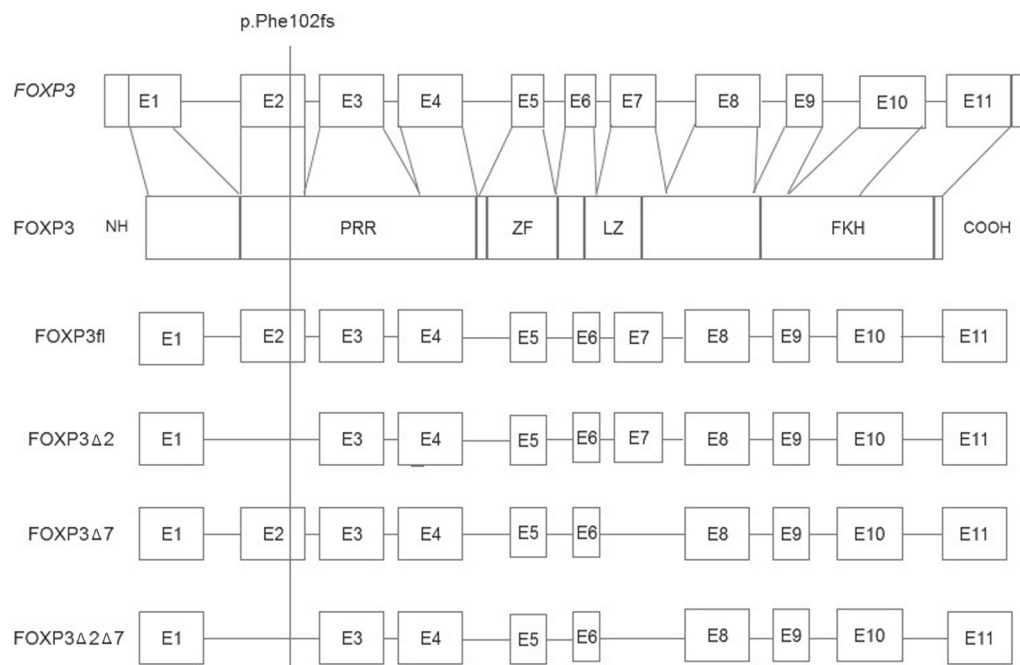


FIG E1. *FOXP3* gene and FOXP3 protein and 4 isoforms. *FOXP3* gene showing 11 coding exons (E1-E11) and FOXP3 protein showing 4 functional domains: a carboxyl terminal forkhead domain; and 3 protein/protein interaction domains, a central zinc finger (ZF), a leucine zipper (LZ), and a proline-rich transcriptional repressor domain (PRR). Four FOXP3 isoforms are also shown: FOXP3fl, the full-length FOXP3 isoform; FOXP3Δ2, the FOXP3 isoform missing the region encoded by exon 2; FOXP3Δ7, an isoform missing the region encoded by exon 7; and FOXP3Δ2Δ7, an isoform missing the region encoded by exons 2 and 7. Finally, the position of a novel p.Phe102fs mutation is shown.

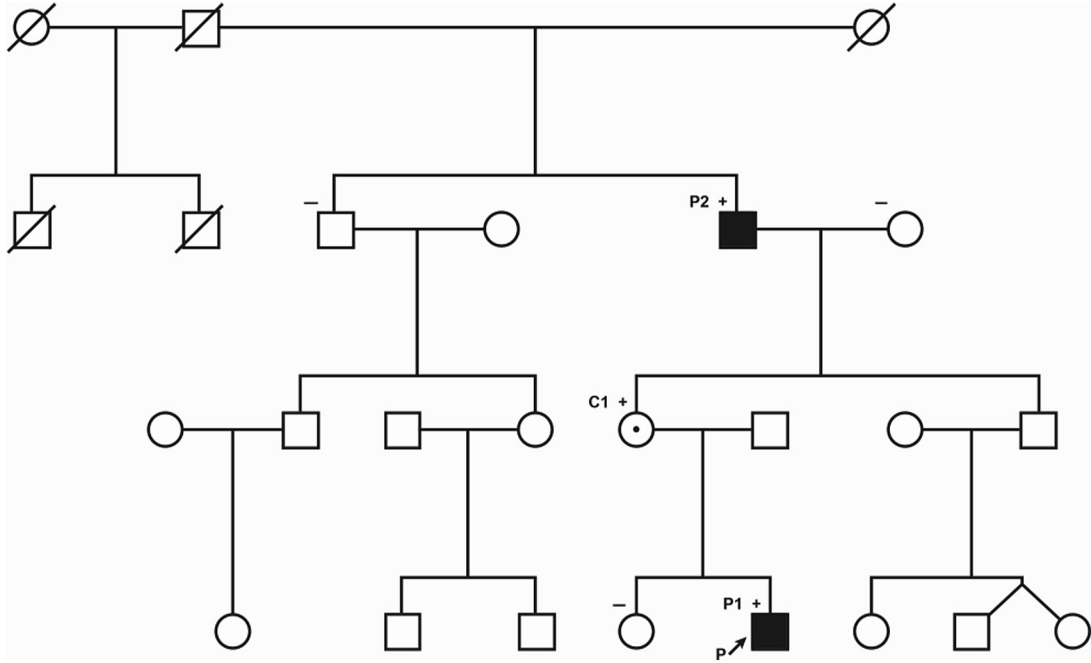


FIG E2. Family pedigree.

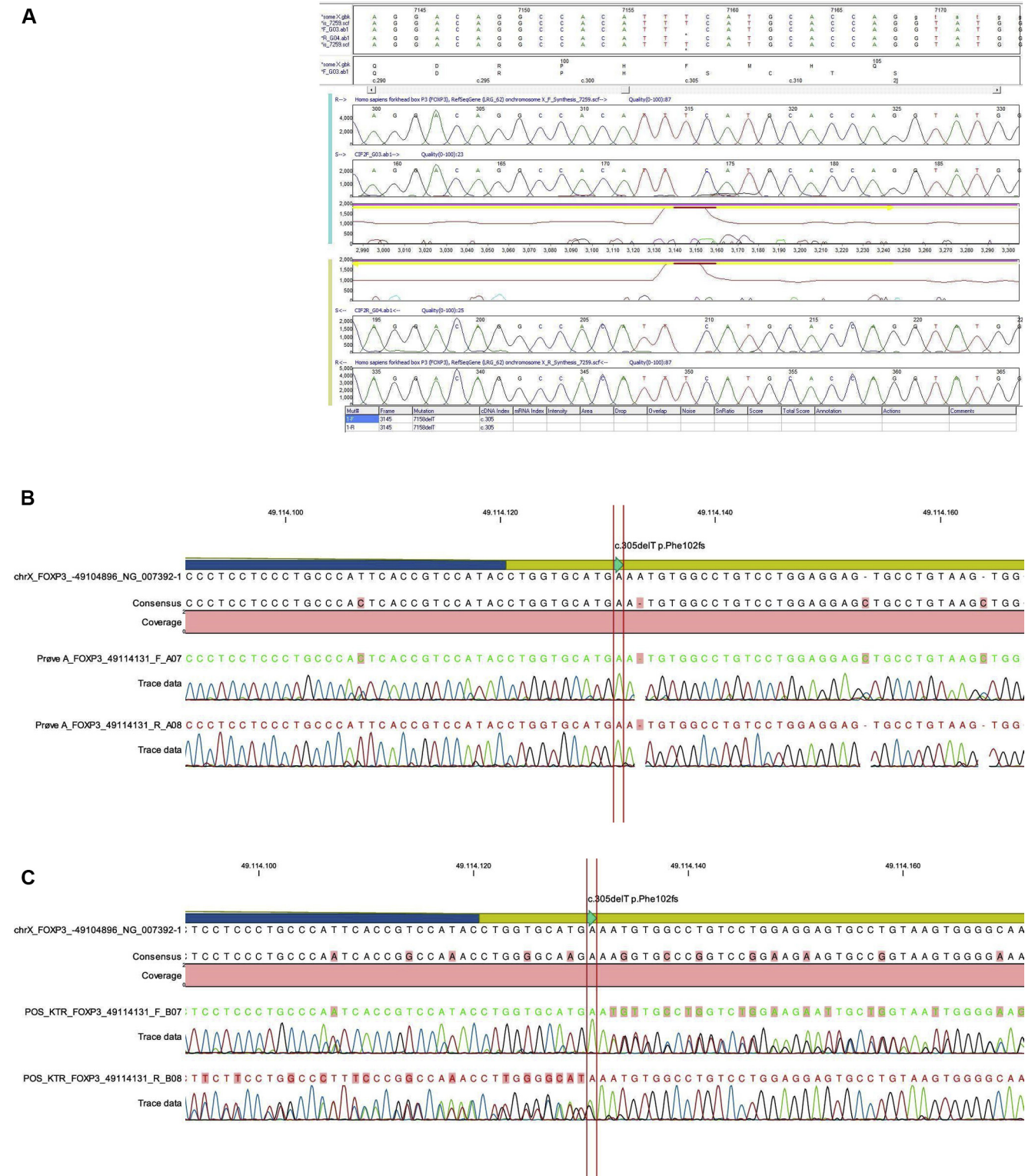


FIG E3. Sanger sequencing of the *FOXP3* gene. **A**, P1 (proband) Sanger sequencing of *FOXP3*. **B**, P2 (proband's grandfather) Sanger sequencing of *FOXP3*. **C**, C1 (proband's carrier mother) Sanger sequencing of *FOXP3*.

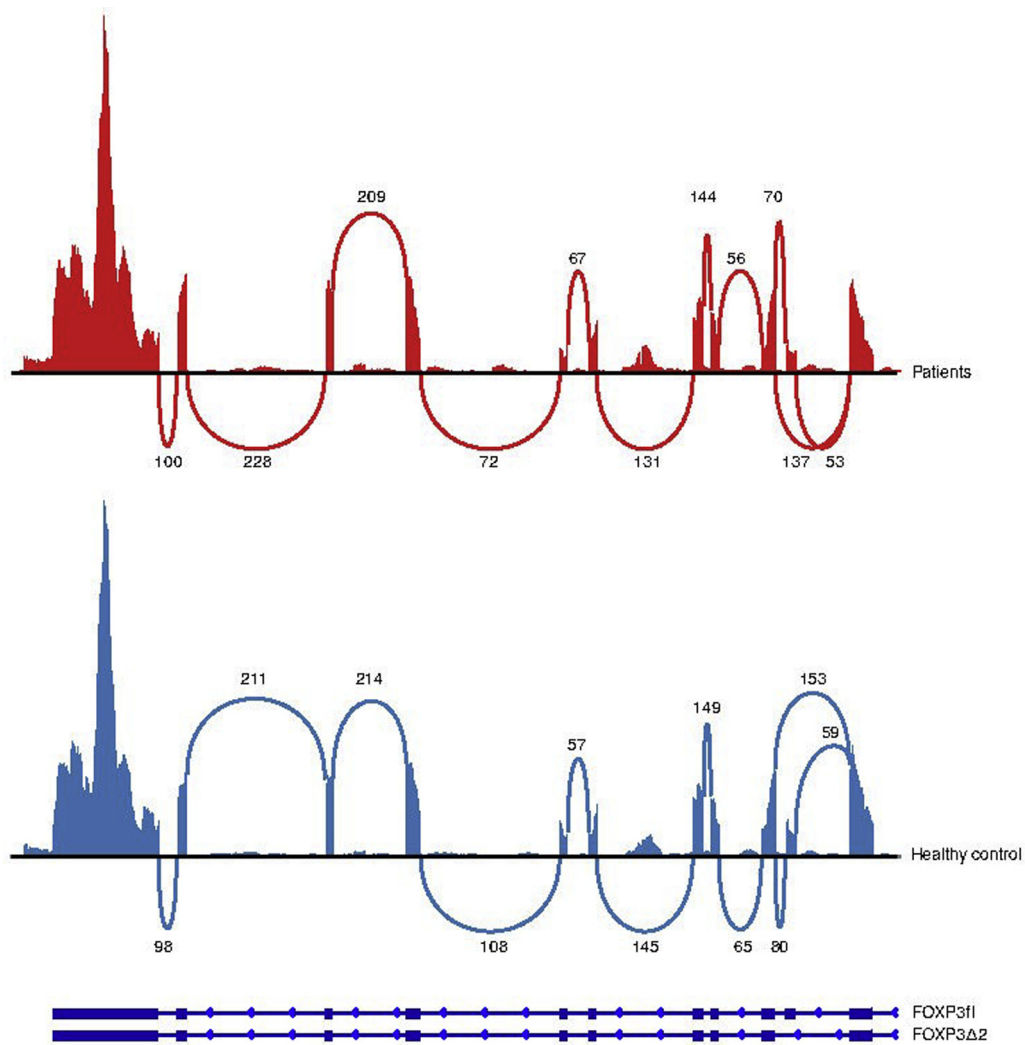
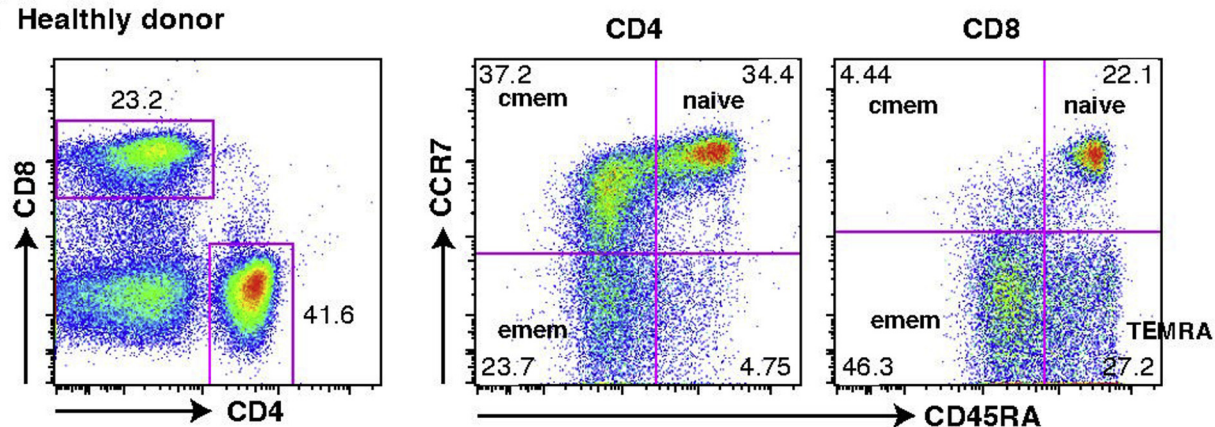
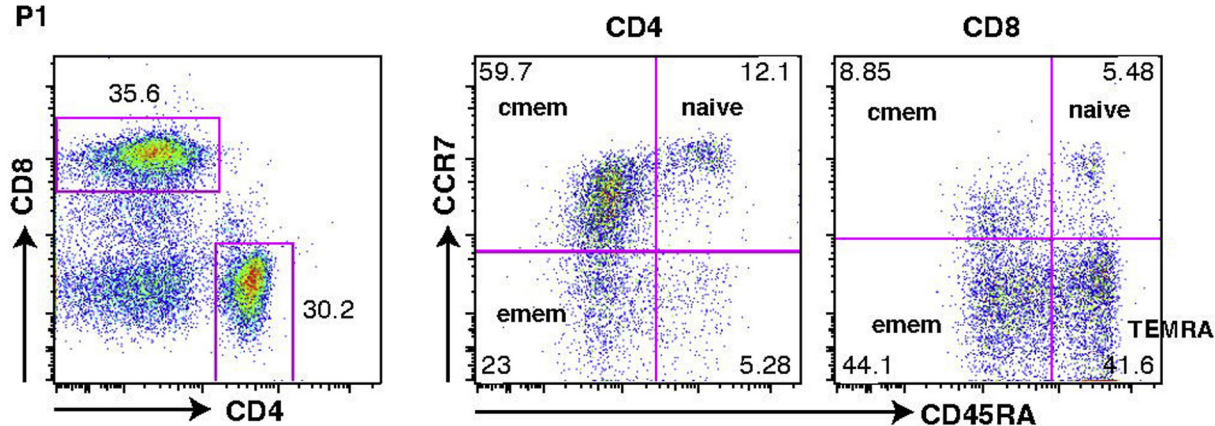


FIG E4. *FOXP3* transcripts in a Sashimi plot from the Integrative Genomics Viewer (IGV) to visualize alternative splicing of *FOXP3* transcripts derived from RNA-sequencing data pooled from the 2 patients with IPEX syndrome (P1 and P2, red) and age-matched control subjects (blue).

A Healthy donor



B P1



C P1

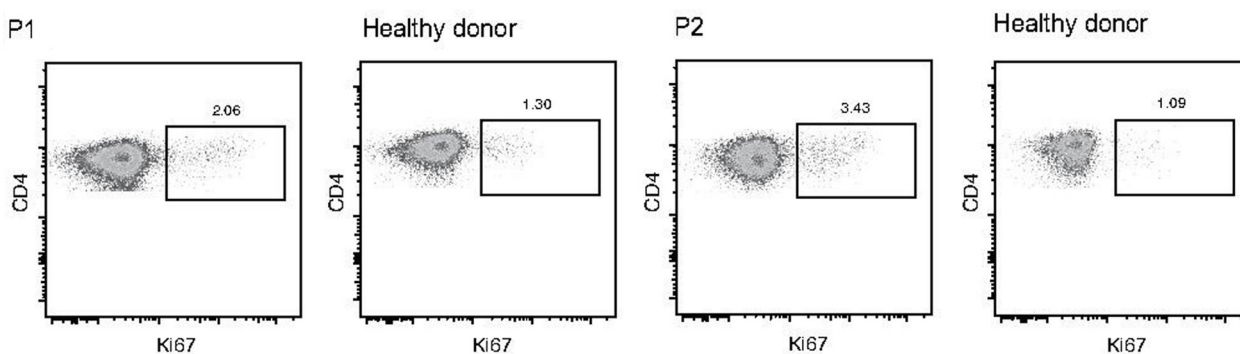


FIG E5. T-cell phenotype of proband (P1). **A** and **B**, Flow cytometric analysis of CD45RA and CCR7 expression on CD4⁺ and CD8⁺ T cells from the proband (P1) compared with a healthy age-matched control subject. **C**, Flow cytometric analysis of Ki67 expression on CD4⁺FOXP3⁻ cells from P1 and P2 compared with age- and sex-matched healthy control subjects.

TABLE E1. Immune results for proband (P1)

IgG (g/L)	14.3 (7-16)
IgM (g/L)	2.4 (0.7-4)
IgA (g/L)	1.3 (0.4-2.3)
IgE (IU)	20 (0-100)
CD3 ($\times 10^9/L$)	1.4 (0.9-1.6)
CD4 ($\times 10^9/L$)	0.7 (0.7-1.2)
CD8 ($\times 10^9/L$)	0.7 (0.3-0.7)
CD16 ⁺ CD56 ($\times 10^9/L$)	0.14 (0.13-0.35)
CD19 ($\times 10^9/L$)	0.2 (0.16-0.36)
<i>Saccharomyces cerevisiae</i> IgA (U/mL)	63
<i>Saccharomyces cerevisiae</i> IgM (U/mL)	70
Gliadin IgA (U/mL)	21 (0-7)
Gliadin IgG (U/mL)	19 (0-7)
Tissue transglutaminase IgA (U/mL)	1 (0-7)
Antinuclear antibodies	Negative
ANCA	Positive
Atypical cANCA	160
Proteinase 3 antibody (EU)	15 (0-5)
Smooth muscle antibodies	Negative
Mitochondrial antibodies	Negative
Gastric parietal cell antibodies	Negative
Liver kidney microsomal antibodies	Negative
Endomysial IgA	Negative
Neutrophil cytoplasm myeloperoxidase antibodies (EU)	1 (0-5)

Boldface indicates abnormal results.

ANCA, Anti-neutrophil cytoplasmic antibody; cANCA, cytoplasmic anti-neutrophil cytoplasmic antibody.

TABLE E2. Patients with *FOXP3* mutations predicted to cause loss of exon 2

	Patient P2	Patient P1	Otsubo et al ^{E2}	Moudgil et al ^{E3}	Gambineri et al ^{E4}	Gambineri et al ^{E4}
Age at onset	40 y	6 mo	5 d	4 mo	Not provided	Not provided
<i>FOXP3</i> gene mutation	c.305delT	c.305delT	227delT	303-304delTT	232_233del	239del
Clinical conditions	Psoriatic arthritis	IDDM UC PSC	Autoimmune thyroiditis Enterocolitis AIHA Renal tubular disorder	Enterocolitis IDDM Hypothyroidism Eczema AIHA Immune complex– mediated MGN	Not provided	Not provided
Treatment		Mesalazine	Tacrolimus Steroid	Cyclosporine Steroid Rituximab MMUD HSCT	Not provided	Not provided

AIHA, Autoimmune hemolytic anemia; *HSCT*, hematopoietic stem cell transplantation; *MGN*, membranous glomerular nephritis; *MMUD*, mismatched unrelated donor; *PSC*, primary sclerosing cholangitis; *UC*, ulcerative colitis.