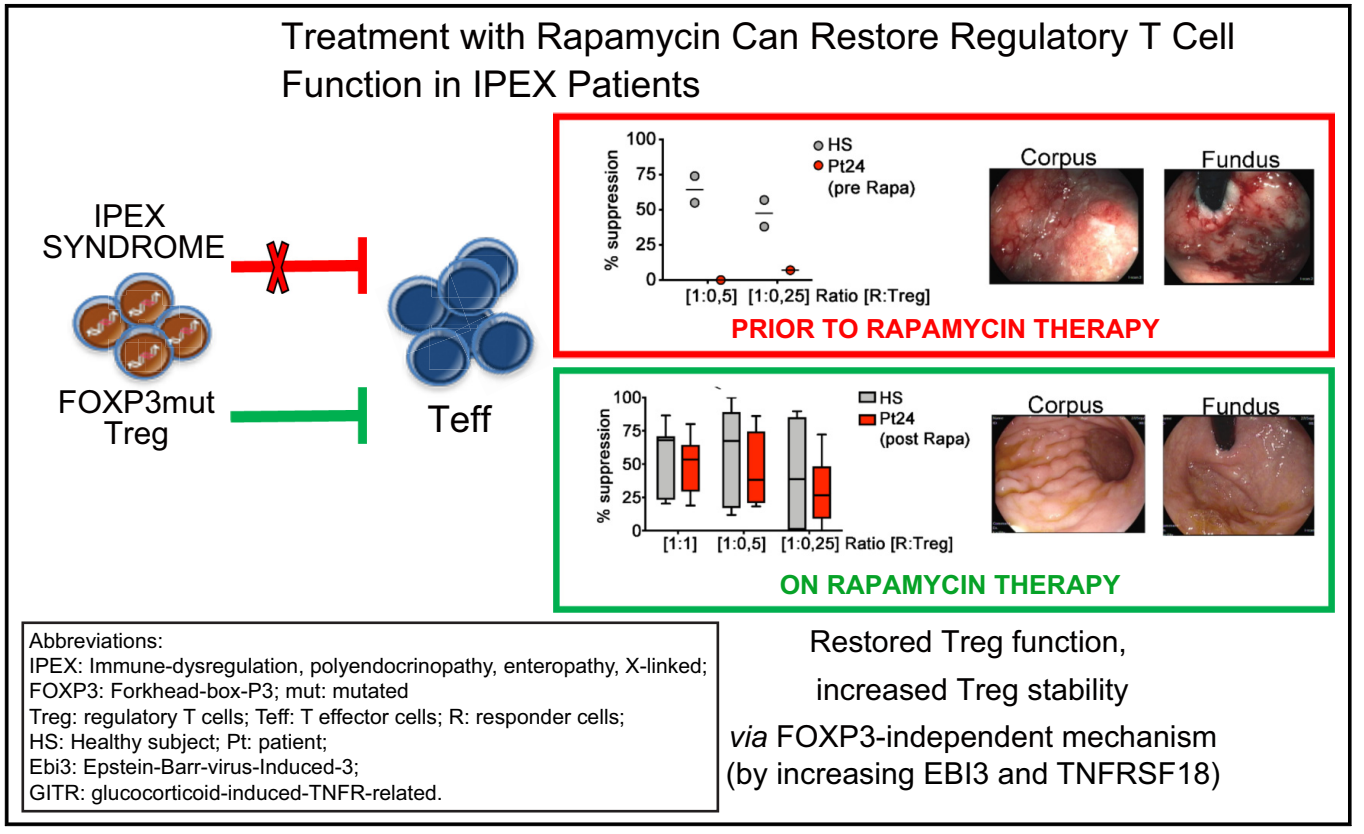
Treatment with rapamycin can restore regulatory T-cell function in IPEX patients

Laura Passerini, PhD,[a]* Federica Barzaghi, MD, PhD,[a,b]* Rosalia Curto, BSc,[a] Claudia Sartirana, BSc,[a] Graziano Barera, MD,[c] Francesca Tucci, MD,[b] Luca Albarello, MD,[d] Alberto Mariani, MD,[e] Pier Alberto Testoni, MD,[e] Elena Bazzigaluppi, BSc,[f] Emanuele Bosi, MD,[f,g] Vito Lampasona, PhD,[f] Olaf Neth, MD,[h] Daniele Zama, MD,[i] Manfred Hoenig, MD,[j] Ansgar Schulz, MD,[j] Markus G. Seidel, MD,[k] Ivana Rabbone, MD,[l] Sven Olek, PhD,[m] Maria G. Roncarolo, MD,[n] Maria P. Cicalese, MD,[a,b] Alessandro Aiuti, MD, PhD,[a,b,g] and Rosa Bacchetta, MD[a,n]

Milan, Bologna, and Turin, Italy, Seville, Spain, Ulm and Berlin, Germany, Graz, Austria, and Stanford, Calif

GRAPHICAL ABSTRACT



From [a]the San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), [b]the Department of Paediatric Immunohematology, [c]the Department of Paediatrics, [d]the Pathology Unit, [e]the Gastroenterology Unit, [f]the Diabetes Research Institute, IRCCS San Raffaele Scientific Institute, Milan; [g]the Vita Salute San Raffaele University, Milan; [h]the Department of Paediatric Infectious Diseases, Rheumatology and Immunodeficiency, Instituto de Biomedicina de Sevilla/Consejo Superior de Investigaciones Científicas/Universidad de Sevilla, Seville; [i]the Department of Pediatrics, S. Orsola-Malpighi Hospital, University of Bologna; [j]the Clinic of Pediatrics and Adolescent Medicine, Ulm University; [k]the Division of Pediatric Hematology/Oncology, Department of Pediatrics and Adolescent Medicine, Medical University Graz; [l]the Department of Pediatrics, University of Turin; [m]the Epiontis GmbH, Berlin; and [n]the Department of Pediatrics, Division of Stem Cell Transplantation and Regenerative Medicine, Stanford University School of Medicine.

*These authors contributed equally to this work.

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Corresponding author: Rosa Bacchetta, MD, Department of Pediatrics, Stanford School of Medicine, 265 Campus Drive, Stanford, CA 94305. E-mail: rosab@stanford.edu.

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Background: Immune-dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a lethal disease caused by mutations in a transcription factor critical for the function of thymus-derived regulatory T (Treg) cells (ie, *FOXP3*), resulting in impaired Treg function and autoimmunity. At present, hematopoietic stem cell transplantation is the therapy of choice for patients with IPEX syndrome. If not available, multiple immunosuppressive regimens have been used with poor disease-free survival at long-term follow-up. Rapamycin has been shown to suppress peripheral T cells while sparing Treg cells expressing wild-type FOXP3, thereby proving beneficial in the clinical setting of immune dysregulation. However, the mechanisms of immunosuppression selective to Treg cells in patients with IPEX syndrome are unclear.

Objective: We sought to determine the cellular and molecular basis of the clinical benefit observed under rapamycin treatment in 6 patients with IPEX syndrome with different *FOXP3* mutations.

Methods: Phenotype and function of *FOXP3*-mutated Treg cells from rapamycin-treated patients with IPEX syndrome were tested by flow cytometry and *in vitro* suppression assays, and the gene expression profile of rapamycin-conditioned Treg cells by droplet-digital PCR.

Results: Clinical and histologic improvements in patients correlated with partially restored Treg function, independent of FOXP3 expression or Treg frequency. Expression of TNF-receptor-superfamily-member 18 (*TNFRSF18*, glucocorticoid-induced TNF-receptor–related) and EBV-induced-3 (*EBI3,* an IL-35 subunit) in patients' Treg cells increased during treatment as compared with that of Treg cells from untreated healthy subjects. Furthermore inhibition of glucocorticoid-induced TNF-receptor–related and Ebi3 partially reverted *in vitro* suppression by *in vivo* rapamycin-conditioned Treg cells.

Conclusions: Rapamycin is able to affect Treg suppressive function via a FOXP3-independent mechanism, thus sustaining the clinical improvement observed in patients with IPEX syndrome under rapamycin treatment. (J Allergy Clin Immunol 2020;145:1262-71.)

Key words: *autoimmunity, Ebi3, FOXP3, GITR, IPEX, mTOR, rapamycin, regulatory T cells, suppression*

Abbreviations used

CTLA-4: Cytotoxic T lymphocyte–associated antigen-4
dd: Droplet digital
Ebi3: EBV-induced-3
FACS: Fluorescence-activated cell sorting
GITR: Glucocorticoid-induced TNF-receptor—related
HS: Healthy subject
IPEX: Immune-dysregulation, polyendocrinopathy, enteropathy, X-linked
IS: Immunosuppressive
LRRC32: Leucine-rich repeat containing protein 32
mTOR: Mammalian target of rapamycin
mTORC: mTOR complex
PB: Peripheral blood
PGK: Phosphoglycerate kinase
R: Responder T cells
t: Thymus-derived
Teff: Effector T
TIGIT: T cell immunoglobulin and ITIM domain
T_{medium}: HS cells without rapamycin
TNFRSF18: Tumor-necrosis-factor-receptor-superfamily-member 18
T_{rapa}: Control (with rapamycin) HS T cells
Treg: Regulatory T
TSDR: Treg-specific demethylated region
wt: Wild-type

FOXP3 is a transcription factor critical for the function of thymus-derived (t) regulatory T (Treg) cells, a subset of $CD4^+$ T lymphocytes devoted to the maintenance of self-tolerance.[1] *FOXP3* mutations result in impaired Treg cell function, the key pathogenic event leading to autoimmunity in immune-dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome.[2] Typically, 90% of patients manifest the disease within the first year of life.[3] The most common symptoms are enteropathy, skin disorders, type 1 diabetes, cytopenia, hepatitis, although milder manifestations, including kidney abnormalities and arthritis, have been reported.[3] Current treatments include supportive and immunosuppressive therapies and allogeneic hematopoietic stem cell transplantation.[2-4] According to the largest reported cohort of patients with IPEX syndrome, hematopoietic stem cell transplantation is the only curative treatment with an overall survival of 73.2% and a stable probability disease-free survival of 56% up to 15 years post hematopoietic stem cell transplantation.[3] In non-transplanted patients, multiple immunosuppressive drugs have been used, including systemic steroids often in conjunction with calcineurin inhibitors, either cyclosporin A or tacrolimus, azathioprine, or less frequently methotrexate/mycophenolate-mofetil, without permanent control of autoimmunity.[3] Rapamycin has been reported to induce clinical remission in several patients with long-term follow-up and its use in IPEX syndrome has become common.[3,5-7]

Rapamycin inhibits the mammalian-target-of-rapamycin (mTOR), a serine-threonine kinase implicated in cell growth, protein synthesis, and proliferation, and is widely used to prevent graft rejection.[8,9] Rapamycin blocks signaling in response to cytokines and growth factors, arrests T-cell cycle progression, and promotes T-cell anergy and tolerance by exerting an immunomodulatory action.[8,9] In murine models, rapamycin facilitates the proliferation/function of tTreg cells and enhances Foxp3 expression.[10] In humans, rapamycin spares and expands $CD4^+CD25^+FOXP3^+$ Treg cells *in vitro*, while inhibiting the proliferation of effector T (Teff) cells.[11] Rapamycin treatment fosters the function of Treg cells in patients with type 1 diabetes[12] and Treg expansion in kidney transplant recipients.[13] Clinical improvement on rapamycin treatment has also been reported in patients with primary immunodeficiencies with prevailing lymphoproliferation, such as autoimmune lymphoproliferative syndrome, activated phosphoinositide 3-kinase δ syndrome, LPS-responsive beige-like anchor protein deficiency, and cytotoxic T lymphocyte–associated antigen-4 (CTLA-4) insufficiency.[14-16] However, to date, the beneficial effect of rapamycin has only been reported for Treg cells expressing wild-type (wt)*FOXP3*.

Here, we have investigated the cellular and molecular impact of rapamycin treatment in Treg cells of patients with IPEX syndrome, to determine whether rapamycin increases the number and/or function of *FOXP3*-mutated Treg cells, and whether this increase is via FOXP3-dependent or -independent mechanisms. Results indicate that the clinical improvement is associated with partial

restoration of Treg function without increase of FOXP3 expression. We propose that rapamycin increases the stability of *FOXP3*-mutated Treg cells, likely by restoring mTORC inhibition lost due to *FOXP3* mutations, and by modulating Treg-specific pathways, such as those mediated by glucocorticoid-induced TNF-receptor–related (GITR) and EBV-induced-3 (Ebi3), thus resulting in better control of autoimmunity.

METHODS

Patients and healthy subjects

IPEX syndrome was diagnosed based on clinical and *FOXP3*-sequencing data. Peripheral blood (PB) was obtained on informed consent, in accordance with the Ospedale San Raffaele (OSR) Ethical Committee (Telethon Institute for Gene Therapy [TIGET] 06/Periblood). Patients with IPEX syndrome recruited under TIGET06 protocol follow internal OSR numbering, previously applied to earlier publications.[17-21] Rapamycin plasma levels were monitored by routine laboratory test.

Rapamycin is administered as oral solution or tablet in children. A usual practice, it has been administered to the patients of the present cohort starting with a loading dose of 2 to 3 mg/m^2 on day 1, followed by a maintenance dose of 1 mg/m^2/day, then adjusted to target the plasmatic level to 8 to 12 ng/mL, given the previous experience in patients with IPEX syndrome.[5-7]

In patient 24, starting from 2 mg/m^2, a rapid increase of the plasmatic concentration was observed 1 month after drug initiation, but rapidly normalized after temporary withdrawal. Rapamycin plasmatic concentrations monitoring have been routinely performed. The timing of monitoring was based on the starting plasmatic level, individual patient features, and stability of the steady-state concentrations. Possible adverse effects include cytopenia, hypercholesterolemia, hypertriglyceridemia, gastrointestinal manifestations, oral aphthous ulcers, acne, arthralgia, and peripheral edema and were periodically monitored as appropriate. No side effects were observed in the present cohort except for patient 24 who developed severe oral aphthous lesions that were dose-dependent and needed temporary drug withdrawal and prednisone administration. As other immunosuppressive agents, rapamycin increases the risk of infections, which were also censored by physical examination and blood tests when necessary. Thus, monitoring of blood test and physical examination was performed in the patients.

Endoscopy and histology

Gut biopsies were fixed in 4% buffered formalin, embedded in paraffin. Then 3-μm sections were stained by routine hematoxylin and eosin by standard histological techniques.

Cell culture

Biopsies were processed as described,[22] and cell suspensions were activated *in vitro* in the presence of plate-bound anti-CD3 10 μg/mL (Miltenyi-Biotec, Bergisch Gladbach, Germany), soluble anti-CD28 1 μg/mL (BD, Franklin Lakes, NJ) mAbs and rhIL-2 100 U/mL (Proleukin; Novartis, Basel, Switzerland). After 10-day expansion, T-cell lines were analyzed by flow cytometry.

PBMCs were purified over lymphoprep density gradient (STEMCELL Technologies, Vancouver, Canada). For suppression assays and mRNA extraction CD4$^+$CD25$^+$CD127^{low} (Treg cells) and CD4$^+$CD25$^-$ (Teff) cells were sorted by fluorescence-activated cell sorting (FACS) (MoFloXDP; Beckman-Coulter, Brea, Calif). All samples were over 95% pure.

For control HS T cells (hereafter T_{rapa}) differentiation, CD4$^+$ T cells were isolated from PBMC by negative selection (Miltenyi-Biotec). CD4$^+$ T cells (>90% pure) were activated with plate-bound anti-CD3 10 μg/mL (Miltenyi-Biotec) and anti-CD28 1 μg/mL (BD) mAbs in the presence or absence of 100 nmol/L Rapamune (Whyet, Madison, NJ) for 3 rounds of stimulation.[11] All cultures were performed in X-VIVO 15/5% human-serum (Lonza, Basel, Switzerland)/penicillin-streptomycin (EuroClone, Pero, Italy).

Cytokine production

To detect intracellular cytokines, lamina propria-derived T-cell lines were activated for 5 hours with phorbol 12-myristate 13-acetate 10 ng/mL plus ionomycin 1 μg/mL in the presence of brefeldin-A 10 μg/mL (all from Sigma-Aldrich).

Flow cytometry

Anti-CD4, anti-CD25, anti-CD62L mAbs (all from BD); anti-T cell immunoglobulin and ITIM domain (TIGIT; eBioscience, San Diego, Calif); anti-CD39, anti-CD127, anti-CD95, and anti-CD4 mAbs (all from BioLegend, San Diego, Calif) were used to prepare antibody cocktails for surface staining. Intracellular staining with anti-CTLA4, anti-FOXP3, anti-Helios (all from BioLegend), and anti-Ki67 (BD) mAbs was performed after fixation/permeabilization with FOXP3-staining kit (eBioscience). Intracellular-cytokine staining was performed after fixation (2% formaldeyde; Thermo Fisher Scientific, Waltham, Mass), and permeabilization (0.5% saponin; Sigma-Aldrich), using an antibody cocktail containing anti-human-IFN-γ (BD), anti-IL-2, anti-IL-17A, and anti-IL-4 mAbs (all from eBioscience). Samples were acquired on BD-FACSCantoII and analyzed with FCS-Express-Pro-Software-4 (De Novo, Glendale, Calif) or FlowJo (BD).

Nucleic acid extraction and PCR

RNA was extracted with RNeasy-kits (Qiagen, Hilden, Germany) and reverse-transcribed with High-Capacity cDNA Reverse-Transcription-Kit (Applied Biosystems, Foster City, Calif). Gene expression was assessed on 20 ng of RNA-equivalent by droplet-digital (dd) PCR (QX200; Bio-Rad Laboratories, Hercules, Calif) with phosphoglycerate-kinase (PGK) used as normalizer of target-gene expression (copies per microliter of the target gene/PGK) and analyzed using QuantaSoft software (Bio-Rad). PGK, FOXP3, leucine-rich-repeat-containing-32 (LRRC32), TGFB1, IL-10, TNF-receptor-superfamily-member-18 (TNFRSF18), Ebi3, and IL-12A mRNAs were quantified with Assay-on-Demand RT-PCR kits (Applied Biosystems). Relative expression is plotted as arbitrary-units (fold-change to the average normalized expression in HS Teff cells).

Suppression assays

FACS-sorted CD4$^+$CD25$^-$responder T cells (R) were labelled with e-fluor-670 proliferation-dye (Invitrogen, Carlsbad, Calif). T cells were activated at a density of 20,000cells/well with anti-CD3/CD28/CD2-coated beads (Miltenyi-Biotec) at a bead to T-cell ratio of 1 to 1. FACS-sorted Treg cells were added at R to Treg cells ratios of 1 to 1, 1 to 0.5 or 1 to 0.25. After a 6-day coculture the percentage of divided precursors was assessed by flow cytometry. Suppression was calculated by comparing the percentage of divided cells in the absence or presence of Treg cells. When indicated, the following mAbs were added: 10 μg/mL anti-human TGF-β1,2,3; anti-human GITR; and anti-human IL-12A mAbs, isotype control (R&D Systems, Minneapolis, Minn); or anti-human Ebi3 mAb (Millipore, Burlington, Mass).

Statistical analyses

Results are presented as median (range) or mean ± SEM, as indicated. Analyses were performed with nonparametric tests (Mann-Whitney/Wilcoxon). *P* less than .05 was considered significant. Statistical analysis was applied to groups of 5 or more independent results.

RESULTS

Clinical and immunological evolution of IPEX pathology in response to rapamycin treatment

We report the clinical follow-up of patient 24 who has IPEX syndrome and who received a 3-year-treatment with rapamycin monotherapy with clinical remission of the disease. Patient 24 was 9 years old when he was referred to our clinic for severe

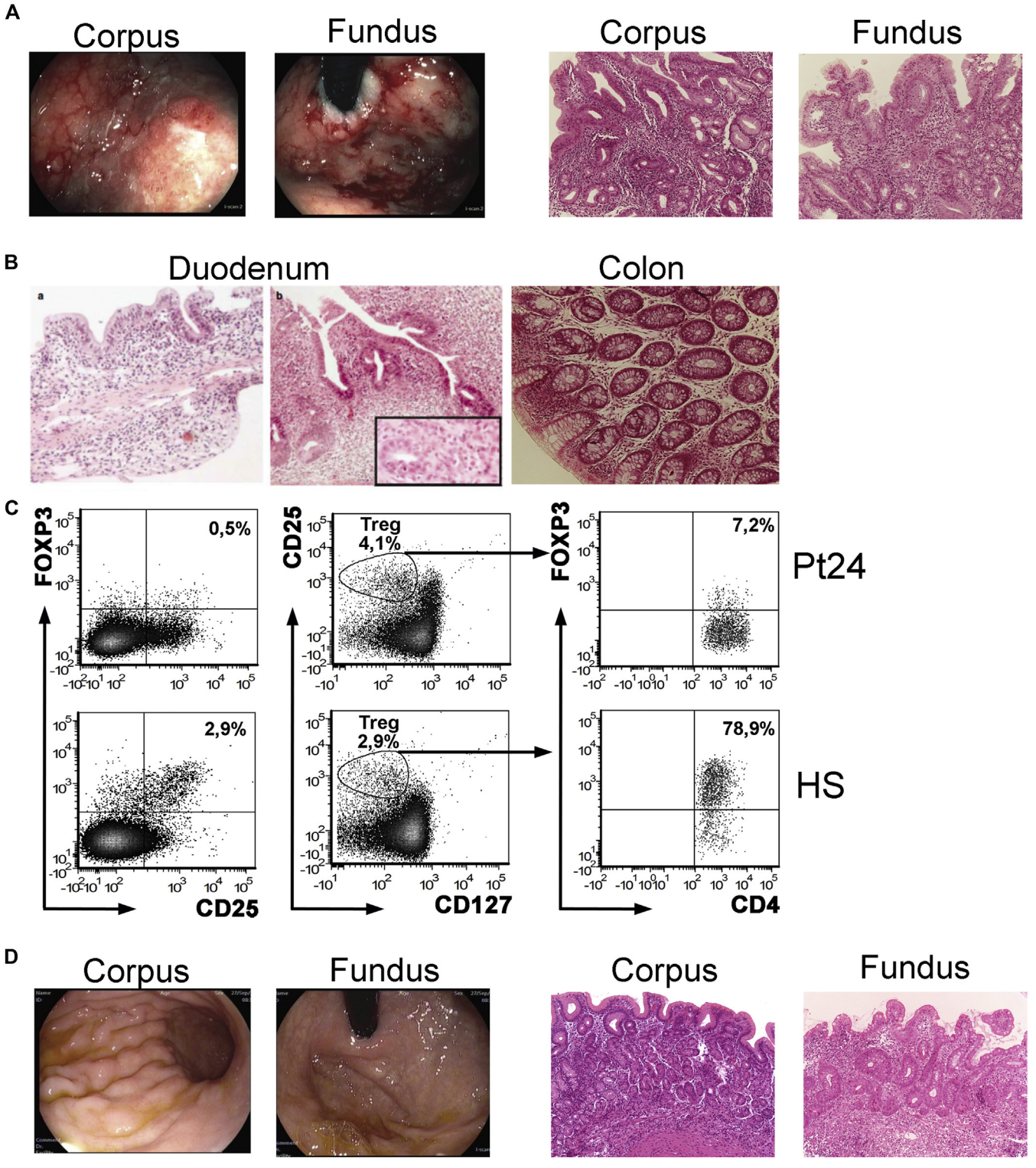

FIG 1. Altered gut histology and reduced FOXP3 expression in IPEX patient with active disease. **A,** Images of corpus/fundus from endoscopy of patient 24 at diagnosis. **B,** Hematoxylin and eosin staining of gut biopsies of patient 24 at diagnosis. **C**, FOXP3 expression in PBMC of patient 24 at diagnosis (*upper*) and of a HS (*lower*). (*Left/middle*) Gate on $CD4^+$. (*Right*) Gate on $CD4^+CD25^+CD127^{low}$. **D**, Images of corpus/fundus from endoscopy of patient 24 after 4 months therapy (*right*) and hematoxylin and eosin staining of gut biopsies of patient 24 on rapamycin therapy (26 months) (*left*).

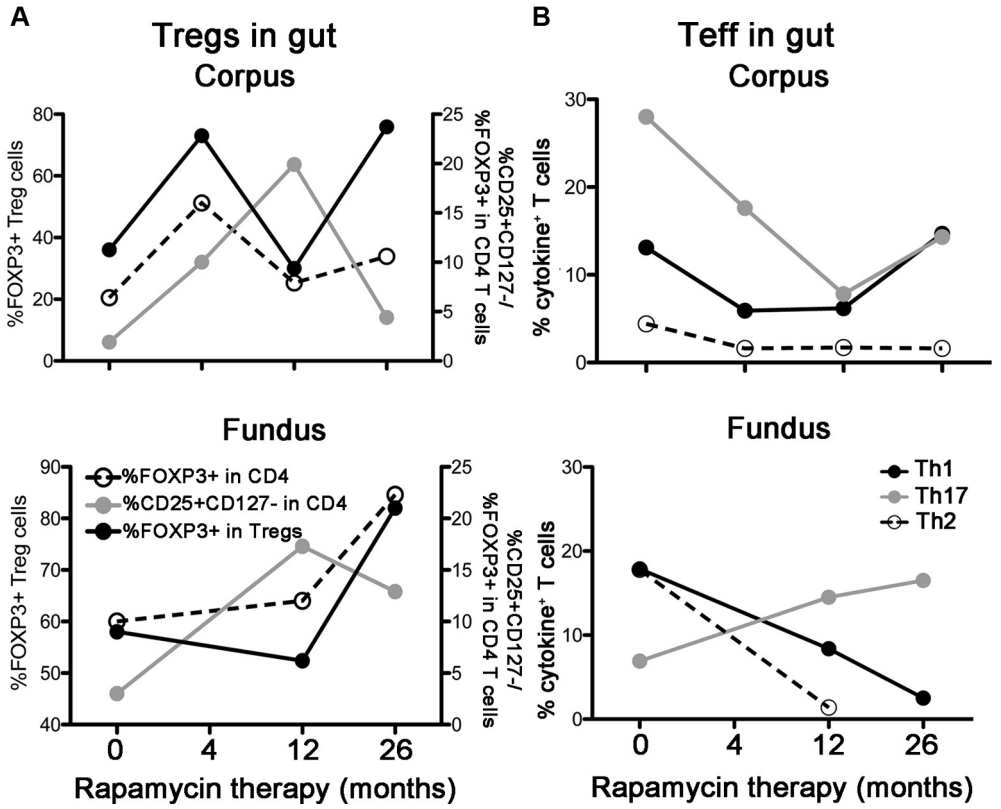

FIG 2. Decreased inflammatory infiltrates in IPEX during therapy with rapamycin. Single-cell suspensions from biopsies (gastric mucosa) of patient 24 were activated with anti-CD3/CD28 mAbs/rhIL-2 for 10 days. **A,** (*right*) The frequency of Treg cells (%FOXP3$^+$ cells—dashed line, %CD25$^+$CD127^{low}—gray line) within CD4$^+$ and (*left*) %FOXP3+ within CD4$^+$CD25$^+$CD127^{low} Treg cells (black filled line). **B,** Intracytoplasmic staining after stimulation with phorbol 12-myristate, ionomycin, and brefeldin A. Black filled line indicates % CD4$^+$/IFNγ$^+$IL-2$^+$IL-17A$^-$IL-4$^-$ (T_H1); dashed line indicates %CD4$^+$/IFNγ$^-$IL4$^+$IL-17A$^-$ (T_H2), and gray line indicates CD4$^+$/IFNγ$^-$IL-17A$^+$IL-4$^-$ (T_H17).

dyspepsia and failure to thrive. Histology showed active inflammatory gastropathy with ulcers and hypotrophic mucosa, diffuse intestinal metaplasia of the glands in the corpus/fundus without morphologic or immunohistochemical signs of *Helicobacter pilori* infection or autoimmune gastritis. Mild duodenal villous hypotrophy and increased intraepithelial lymphocytes were observed, but minimal involvement of colon (Fig 1, *A* and *B*). A dysimmune origin was suggested. Suspicion of IPEX syndrome was raised based on high titer of anti-harmonin autoantibodies (>100 U)[20] and low FOXP3 expression within the peripheral CD4$^+$CD25$^+$CD127^{low} T cells (Fig 1, *C*). *FOXP3* sequencing revealed a mutation at exon+1 splice junction (c.210+1G>C),[3] resulting in aberrant mRNA splicing isoforms (see Fig E1 in this article's Online Repository at www.jacionline.org) and aberrant protein expression (see Fig E2 in this article's Online Repository at www.jacionline.org). We started rapamycin treatment (target plasma level: 8-12 ng/mL); steroids were added to treat aphthosis at occurrence. On treatment, growth and nutritional indexes rapidly increased. The T-cell infiltrate of the gut mucosa decreased and improvement of the lesions was visible 4 months after (Fig 1, *D*). Lymphocytes infiltrating the gut mucosa were isolated and characterized prior to and during therapy (Fig 2, *A* and *B*). The frequency of Treg cells infiltrating the lamina propria of the corpus/fundus increased during therapy, both in terms of CD4$^+$CD25$^+$CD127$^-$ T cells and of FOXP3 expression (Fig 2, *A*), while the frequency of Teff cells, as determined by cytokine production after short-term *in vitro* expansion of infiltrating T cells, drastically decreased over time, except for residual T_H17 cells in the fundus (Fig 2, *B*). The regulatory skew of the T-cell infiltrate (Fig 2, *A*) correlated with progressive histologic reduction of the mucosal inflammation (Fig 1, *D*). Stable recovery of tissue architecture was achieved at 26 months of treatment, although residual patches of hypotrophic mucosa were present (not shown).

Rapamycin treatment does not result in increased frequency of circulating FOXP3-mutated Treg cells

We monitored the frequency of Treg cells in the PB of patient 24 after initiation of rapamycin treatment (Fig 3). Unlike that observed in the gut tissue, the frequency of peripheral FOXP3$^+$ T cells remained low on treatment (Fig 3, *A*), and there was no correlation in increased FOXP3 expression with rapamycin plasma levels (Spearman correlation $P > .05$) (see Fig E3 in this article's Online Repository at www.jacionline.org). Accordingly, the sporadic peaks of FOXP3-expressing T cells were not due to expansion of tTreg cells, as demonstrated by constant frequency of Treg cell-specific demethylated region (TSDR)-demethylated cells in PB (see Fig E4 in this article's Online Repository at www.jacionline.org). Furthermore, *in vivo* Treg cell proliferation, measured by the proliferation-marker Ki67, was not increased

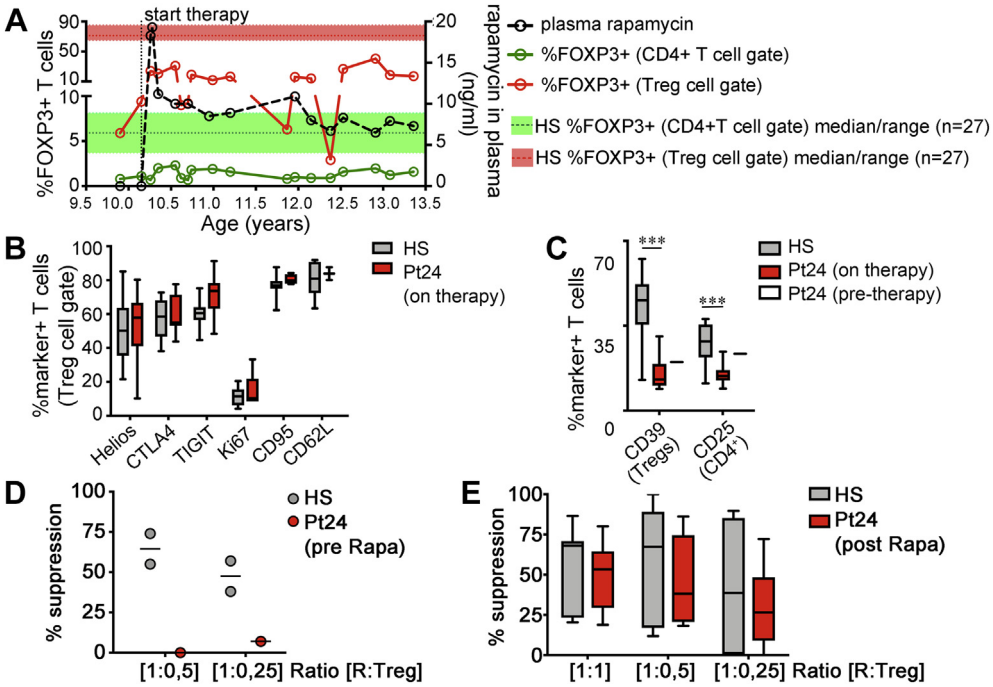

FIG 3. FOXP3 expression in circulating Treg cells during rapamycin therapy in IPEX syndrome. **A,** Frequency of Treg cells in PB of patient (*Pt*) 24 before and during therapy: green line indicates %FOXP3+ and red line indicates %CD4$^+$CD25$^+$CD127^{low} (within CD4$^+$). Shaded areas are the median values and range in HSs (n = 27). The age of patient 24 is plotted. **B,** Expression of the Treg cell-associated markers Helios, CTLA4, TIGIT, CD95, and CD62L and the proliferation marker Ki67 was assessed at multiple time points during therapy (HSs: n ≥ 10; patient 24: n = 2-16, gate CD4$^+$CD25$^+$CD127^{low}, median [range], *P* = ns). **C,** The %CD39$^+$ within CD4$^+$CD25$^+$CD127^{low} and %CD25$^+$ within CD4$^+$ prior to and at multiple time points during therapy (HSs: n ≥ 10; patient 24: pretherapy n = 1; on-therapy n = 18, median [range]; ***P < .001, Mann-Whitney). **D,** The %suppression of Treg cells from HSs (n = 2) and patient 24 pretherapy (R to Treg cell ratio = 1:0.5: %suppression Pt Treg cells: 0, n = 1, HS Treg cells median [range]: 64.5 [55%-74%], n = 2; 1:0.25: %suppression Pt Treg cells: 7, n = 1; HS Treg cells: 47.5 [39%-57%], n = 2). **E,** The %suppression of Treg cells from HSs and patient 24 on-therapy (Pt Treg cells median [range]: 1:1: 53.4 [18.9%-80.0%], n = 9; 1:0.5: 38.2 [18.2%-86.1%], n = 6; 1:0.25: 26.6 [0.0%-72.2%], n = 6; HS Treg cells: 1:1: 68.0 [20.4%-86.5%], n = 9; 1:0.5: 67.4 [11.9%-100%], n = 6; 1:0.25: 38.7 [0.0%-89.6%], n = 6). *P* = ns, Mann-Whitney.

(Fig 3, *B*). The expression of Treg cell-associated markers, including Helios, CTLA4, TIGIT, CD95, CD62L, was comparable to that detected in HS Treg cells (Fig 3, *B*), with the exception of CD39 ($P = .0004$, HSs n = 10 vs patient 24 n = 9, Mann-Whitney) (Fig 3, *C*) and CD25, whose expression was low during therapy ($P < .0001$, HSs vs patient 24 n = 18, Mann-Whitney) (Fig 3, *C*). Overall, these data indicate that rapamycin resulted in enriched Treg cells in the target tissue, but did not increase the frequency of peripheral *FOXP3*-mutated Treg cells, nor induced mutant FOXP3 expression.

Rapamycin treatment restores the suppressive function of FOXP3-mutated Treg cells

At diagnosis and before any treatment, the CD4$^+$CD25$^+$CD127^{low} Treg cells of patient 24 did not show suppressive function *in vitro* (Fig 3, *D*). The assay was repeated in 9 independent assays from 2 months to 3 years after initiation of rapamycin therapy at different R to Treg cell ratios (1:1, n = 9; 1:0.50, n = 6; 1:0.25, n = 6). Although the overall suppressor potency was reduced as compared to that of HS Treg cells, rapamycin stably restored Treg cell function (Fig 3, *E*). Data were also confirmed when patient 24's CD4$^+$CD25$^-$ T cells were used as Rs (see Fig E5 in this article's Online Repository at www.jacionline.org).

We further analyzed Treg cells from 5 patients with IPEX syndrome under rapamycin treatment (Fig 4, and see Table E1 in this article's Online Repository at www.jacionline.org). Unlike for patient 24, for all other patients rapamycin was not the only immunosuppressive (IS) treatment, but rapamycin was administered while tapering alternative IS drugs (see Table E1 for concomitant medications at time of sampling). Although only tested during treatment, the frequency of peripheral Treg cells, measured as CD4$^+$CD25$^+$CD127^{low} T cells, was comparable to that measured in HS, whereas expression of intracellular FOXP3 was highly variable ($P > 0.5$, Mann-Whitney) (Fig 4, *A* and *B*, and see Table E2 in this article's Online Repository at www.jacionline.org), likely depending on FOXP3 mutation site (Table E1). Similar to findings in patient 24's PBMCs (Fig 3, *C*), expression of CD25 in CD4$^+$ T cells was lower in patients under therapy than in HSs ($P < .05$, Mann-Whitney) (Fig 4, *C*, Table E2), likely due to reduced frequency of activated CD4$^+$ T cells. In contrast, CD39, whose expression remained impaired in patient 24 (Fig 3, *C*), was higher in this cohort of patients than in HSs

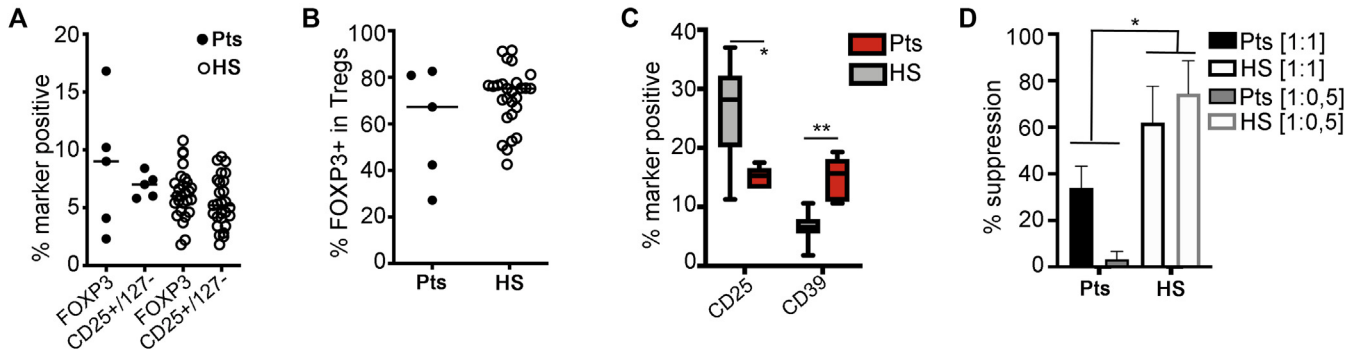

FIG 4. Treg cell frequency and function in patients with IPEX syndrome under rapamycin therapy. The % $CD4^+CD25^+CD127^{low}$ and $\%FOXP3^+$ within $CD4^+$ (**A**) and $\%FOXP3^+$ within $CD4^+CD25^+CD127^{low}$ (**B**) in PB of patients on-therapy (n = 5) and HSs (n = 27); *P* = ns, Mann-Whitney. **C,** The %CD25/CD39 in the indicated cell-gate. **P* < .05, ***P* < .01 versus HSs (n = 16), Mann-Whitney. **D,** The %suppression of Treg cells from HSs and patients at R to Treg cell ratios of 1:1 or 1:0.5. Bars and errors indicate median and range; dots represent single assays. The %suppression median [range] at 1:1 ratio: patients: 34 [12-57], HSs: 59 [33-98], n = 4; at 1:0.5 ratio: patients 0 [0-10], HSs 72 [52-100], n = 3; **P* < .05, Wilcoxon paired-test.

(*P* < .01, Mann-Whitney) (Fig 4, *C*, Table E2), in line with the known effect of rapamycin on CD39.[23] We tested the suppressive ability of Treg cells from 4 of 5 treated patients with IPEX syndrome (patients 25, 28, 30, and 31) at R to Treg cell ratio of 1:1 and 1:0.5 (Fig 4, *D*). We found that some level of suppressive activity was detectable in most patients, although their function was significantly lower than that detected in HSs (*P* = .02, Wilcoxon paired-test) (Fig 4, *D*). These results indicate that *FOXP3*-mutated Treg cells exposed to rapamycin *in vivo* display suppressive activity *in vitro*, which might sustain the clinical improvement observed in these patients.

Rapamycin expands functional FOXP3-mutated Treg cells *in vitro*

It has been shown that a culture of activated wtFOXP3 $CD4^+$ T cells in the presence of rapamycin can prevent expansion of Teff cells and promote the survival of Treg cells.[11] To test this effect with mutant FOXP3, $CD4^+$ T cells from 3 patients with IPEX syndrome who were not under rapamycin treatment at time of sampling (patients 20,[19,20] 24, and 26) were cultured in the presence of rapamycin. After *in vitro* expansion, as expected, T_{rapa} were enriched for FOXP3-expressing cells and displayed efficient suppression, whereas those expanded without rapamycin (T_{medium}) did not (see Fig E6, *A* and *B* in this article's Online Repository at www.jacionline.org). In 2 (patients 20 and 26) of the 3 patients with IPEX syndrome tested, FOXP3-expressing cells were increased in T_{rapa} cultures (Fig E6, *A*). T_{rapa} from patient 24, with the splice-site mutation that prevents FOXP3 expression (Fig 1, Fig E1), did not upregulate FOXP3-expressing cells (Fig E6, *A*). However, T_{rapa} of all the patients acquired suppressive function, although less potently than that observed by wt*FOXP3* T_{rapa}, in line with results from *ex vivo* Treg cells (Fig E6, *B*).

These data suggest that the acquisition of suppressive function by Treg cells after expansion in the presence of rapamycin is independent of the mutated *FOXP3* residual expression.

Rapamycin upregulates Ebi3 and GITR expression in FOXP3-mutated Treg cells

To test the hypothesis that rapamycin-mediated restoration of the suppressive function in *FOXP3*-mutated Treg cells, is via a direct impact on key Treg cell-associated molecules,[24] we analyzed the expression of various genes by ddPCR using FACS-sorted $CD4^+CD25^+CD127^{low}$ Treg cells and $CD4^+CD25^-$ Teff cells from patients with IPEX syndrome under rapamycin treatment (n = 6 samples from 4 patients: patients 24, 27, 28, and 31) and HSs (n = 7) (Fig 5, *A*, and see Tables E3 and E4 in this article's Online Repository at www.jacionline.org). FOXP3 mRNA was highly expressed by IPEX Treg cells, as in HS Treg cells. As expected, LRRC32 (glycoprotein A repetitions predominant) was preferentially expressed by Treg cells, where it contributes to Treg cell-mediated suppression by binding latent-TGFβ on activated Treg cells,[24] with no alterations in patients Treg cells (Treg cells vs Teff cells: *P* < .05) (Fig 5, *A*, Tables E3 and E4). TGF-β1 mRNA was expressed at comparable levels in both Treg cells and Teff cells from HSs and patients. In addition, IL-10 mRNA was preferentially expressed by Treg cells both in patients and HSs (Fig 5, *A*, Tables E3 and E4).

The mRNAs for 2 proteins—GITR and Ebi3—showed significant increases when comparing Treg cells of HSs and patients with IPEX syndrome. While HS Treg cells expressed 3-fold higher GITR mRNA (TNFRSF18, considered a marker of active Treg cells[24]) than Teff cells (*P* < .05), the rapamycin-conditioned IPEX Treg cells displayed significant upregulation of GITR mRNA versus HS Treg cells (*P* < .01), suggesting contribution of this molecule to Treg cell-suppression during treatment. In addition, the 2 subunits of the immunosuppressive cytokine IL-35—Ebi3 and p35/IL-12A—were also analyzed. Expression of IL-12A was equally enriched in Treg cells from both patients with IPEX syndrome and HSs. In contrast, Ebi3 mRNA was significantly higher in both Treg cell- and Teff cell-derived IPEX samples, as compared to HS samples, with a 6-fold increase in rapamycin-conditioned versus HS Treg cells (*P* < .01 Mann-Whitney) (Fig 5, *A*, Table E3). However, the overall expression of IL-35 subunits was low in all samples tested (Table E4). Of note, when Ebi3 and GITR mRNA expression was measured in Treg cells and Teff cells from 2 patients with IPEX syndrome who were not under rapamycin therapy (patients 29 and 30), expression was comparable to that observed in HSs (see Fig E7 in this article's Online Repository at www.jacionline.org). In summary, expression of Ebi3 and GITR

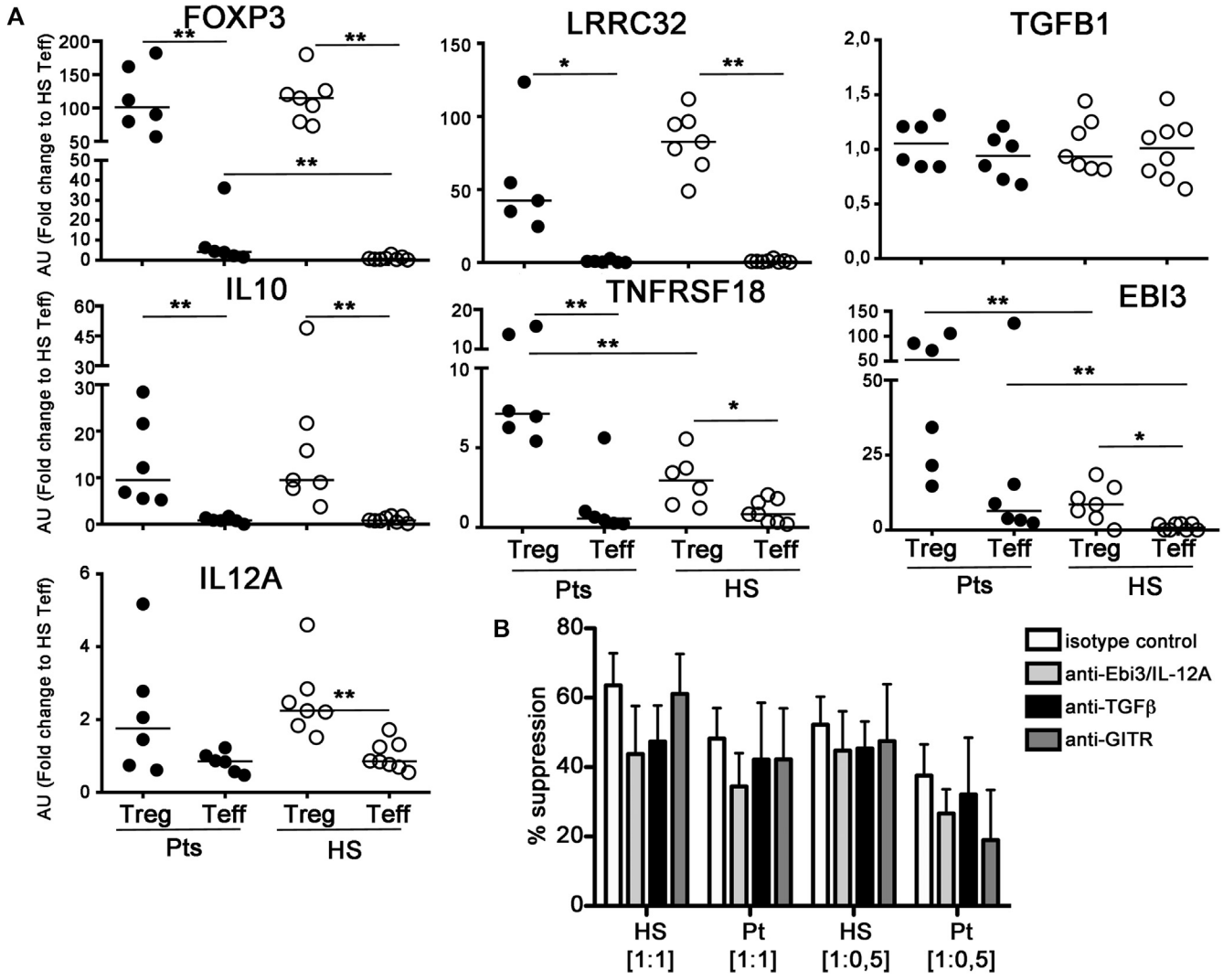

FIG 5. Rapamycin modulates IPEX Treg cell expression profile. **A.** The expression of FOXP3/TGFB1/LRRC32/IL-10/TNFRSF18/EBI3/IL-12A mRNAs by ddPCR on FACS-sorted Treg cells and Teff cells (n = 6 samples from n = 4 patients—patients 24, 27, 28, and 31; HSs n = 7). $^{**}P < .01$; $^{*}P < .05$, Mann-Whitney. Arbitrary units (*AU*): fold-change to average in HS Teff. **B,** The %suppression of Treg cells from HSs/patients on-therapy in the presence of anti-TGF-β1,2,3 (black), anti-Ebi3/IL-12A (light gray), anti-GITR (dark gray) mAbs, or isotype-control (white). Mean ± SEM: R to Treg cell ratio = 1:0.5: isotype control: 37.6 ± 8.9%, n = 5; anti-Ebi3/IL-12A: 26.6 ± 7.0%, n = 4; anti-GITR: 18.9 ± 14.5%, n = 3. HSs n = 4-6; patients n = 3-5 independent experiments on 2 patients on-therapy (patients 24 and 28).

(TNFRSF18) increases during rapamycin treatment in Treg cells of patients with IPEX syndrome, which may contribute to the partial recovery of Treg cell function.

We further tested the expression of IL-35 subunits and GITR mRNA in $T_{rapa/medium}$[11] from HSs (n = 5). T_{rapa} expressed 2.5-fold more Ebi3 and 3-fold more IL-12A than T_{medium}. On the contrary, GITR mRNA expression was not upregulated in HS T_{rapa} (see Fig E8 in this article's Online Repository at www.jacionline.org). Therefore, while IL-35 subunits were upregulated on rapamycin treatment in both *FOXP3*-mutated and wt T cells, GITR upregulation was a specific effect of *in vivo* treatment on IPEX Treg cells.

Blockade of Ebi3/p35 and GITR reverts the suppressive activity of rapamycin-conditioned FOXP3-mutated Treg cells

We tested the effect of GITR and IL-35 blockade during *in vitro* suppression assays using multiple Treg cell samples from patients 24 and 28 (Fig 5, *B*). At R to Treg cell ratio of 1:1, no significant alteration of suppression was detected using both HS Treg cells (n = 4-6) and rapamycin-conditioned Treg cells from patients (n = 3-5 independent experiments), except for a nonsignificant reduction of the suppressive ability of Treg cells in both HSs and patients in the presence of anti-Ebi3/IL-12A mAbs ($P > .05$, Wilcoxon paired test). At R to Treg cell ratio of 1:0.5,

the suppression of HS Treg cells in the presence of blocking antibodies was still comparable to that detected in control cultures, whereas in the presence of anti-Ebi3/IL-12A and anti-GITR-mAbs rapamycin-conditioned Treg cells from patients with IPEX syndrome lost from 30% to 50% of their *in vitro* inhibitory capacity, respectively (n = 3-4, P = ns). In the same conditions, addition of anti-TGF-β mAb did not affect Treg cell function.[10] These data suggest that, among the Treg cell-associated molecules we tested, Ebi3/IL-12A and GITR mainly contributed to improve suppressive ability of FOXP3-mutated Treg cells during rapamycin treatment.

DISCUSSION

We investigated the effects of rapamycin on Teff cells and Treg cells carrying different *FOXP3* mutations, all causative for IPEX syndrome. Our study started from a detailed monitoring of the response to rapamycin monotherapy in 1 patient, followed by validation of the results in additional patients undergoing rapamycin therapy. We found that the T-cell inflammatory infiltrate in the gut decreased during rapamycin therapy in favor of a regulatory-like T-cell infiltrate, a finding that correlated with tissue healing and clinical improvement. Concomitantly, *FOXP3*-mutated Treg cells that had been conditioned by rapamycin acquired suppressive function, independent of whether there was residual FOXP3 expression. In addition, we observed an upregulation of the immunomodulatory proteins GITR (TNFRS18) and Ebi3 that we found to affect the restored suppressive function in IPEX Treg cells. We propose that the beneficial effect of rapamycin in the treatment of IPEX relies on both the inhibition of Teff cells and the stabilization of Treg cell phenotype and function, and that this benefit is independent of *FOXP3*.

Rapamycin inhibits the mTORC1 complex, which regulates cell growth and metabolism in response to environmental signals, resulting in decreased proliferation/differentiation of Teff cells.[25] Accordingly, we report inhibition of mucosal T cells from gastric biopsies of a patient with IPEX syndrome under rapamycin monotherapy. The immunomodulatory effect of rapamycin was also evident in PB, where we found reduced CD25 expression on $CD4^+$ T cells in all patients, indicating reduced activation of Teff cells. Decreased inflammation was associated with increased Ebi3 expression by Teff cells. Murine EBI3 is a negative regulator of IL-17 expression.[26] We speculate that Ebi3 upregulation may contribute to both the dampening of Teff cells, including T_H17 cells in the gut tissue, and the increase in tissue Treg cells in the target organ.

Unlike other immunosuppressants, rapamycin can promote Treg cell expansion,[11,27] although the underlying mechanism is not fully defined. This protolerogenic effect of rapamycin has been attributed to increased expression of wtFOXP3, leading to acquisition of suppressive function. Other studies have reported that stable inhibition of mTORC1 activity in Treg cells is dependent on FOXP3 via the Smgs1-PP2A pathway.[28] Thus, *FOXP3* mutations may reactivate mTORC1 in Treg cells with consequent decreased suppressive function, as reported in PP2A knockout.[28] In line with our results, recently published work by Charbonnier et al.[29] demonstrates that Foxp3 deficiency dysregulates mTORC2 signaling and metabolic pathways in Treg cells and that mTOR inhibitors restored suppressive function in Foxp3-deficient Treg cells by enforcing a metabolic switch that suppresses the Teff-like reprogramming of dysfunctional Treg cells. Although further studies are needed to dissect the underlying molecular mechanism, our data support the hypothesis that rapamycin reestablishes mTORC inhibition in *FOXP3*-mutated Treg cells, thus compensating for FOXP3 dysfunction and partially reverting dysregulation.

CD39 is an ectoenzyme that dephosphorylates ATP to form AMP, thus contributing to the adenosine-mediated inhibitory function of Treg cells.[30] The reported enhancing effect of rapamycin on CD39 expression in HS[23] was confirmed in all patients tested except in patient 24, whose mutation abrogated full-length FOXP3 expression, suggesting that restored Treg cell function under rapamycin treatment may involve multiple mechanisms.

Our data support a specific function for GITR and Ebi3 in the beneficial effects observed in rapamycin-conditioned *FOXP3*-mutated Treg cells. First, we find an increase in GITR expression, which leads to an activated or more stable Treg cell phenotype during therapy. Second, there is a consistent increase of Ebi3-encoding mRNA in T cells of patients under rapamycin treatment. While there is considerable literature highlighting the suppressive potential of the Ebi3-containing cytokine IL-35 in mouse models,[31,32] comparable validation for the human counterpart is limited. Earlier studies failed to detect IL-35 production by human Treg cells.[33] Later studies highlighted that IL-35 is associated with maximal Treg cell function or may mark highly suppressive human Treg cells.[34] Our results suggest that, although absolute Ebi3 mRNA levels were low, and no IL-35 cytokine was detected in PBMC supernatants (not shown), the upregulation of Ebi3 may play a functional role in the beneficial effects observed. We conclude that the Ebi3 subunit either alone, or as part of a yet unidentified cytokine, is required for the rapamycin-mediated immunomodulatory effect observed in Treg cells. Based on the reported Ebi3-dependent induction of IL-17,[26] rapamycin may improve the stability of Treg cells by inhibiting the T_H17-skew of unstable Treg cells, a switch that has been observed when wtFOXP3 is absent.[19]

In conclusion, rapamycin partially compensates for FOXP3 dysfunction in IPEX, by modulating Teff cells and empowering multiple pathways in Treg cells, including restoring mTORC1 inhibition and upregulating GITR and Ebi3, thus leading to improved suppression and control of autoreactive cells.

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Clinical implications: Rapamycin therapy allows clinical, histologic, and immunologic improvement in patients with IPEX syndrome, supporting its preferential use to overcome their Treg cell dysfunction.

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METHODS

PCR

To detect different FOXP3 mRNA isoforms, nonquantitative RT-PCR was performed using cDNA from activated PBMCs of patient 24. Primer pairs were designed spanning different coding regions (sequences available on request): exons+1 to 3 (primer pair 1); exons−1 to 5 (primer pair 2); exons+2 to 5 (primer pair 3); exons+5 to 10 (primer pair 4) (Fig E1). PCR products were run onto 2% Agarose gels (EuroClone, Pero, Italy) to assess amplicon length and sequenced by Sanger sequencing (Primm, Milan, Italy) to reconstruct the mutated isoforms.

Western blotting

Cells were lysed in lysis buffer (1% SDS, 10 mmol/L HEPES, and 2 mmol/L EDTA pH 7.4), heated at 95°C for 5 minutes and sonicated. Proteins (10 μg per lane) were separated onto NuPAGE (4%-12%) Bis-TRIS/3-Morpholinopropane-1-sulfonic acid (MOPS; Thermo Fisher Scientific) and transferred onto nitrocellulose filters (Bio-Rad). After blocking in TRIS-buffered saline with 0.05% Tween 20 plus 5% nonfat dried milk, membranes were probed with mouse anti-human FOXP3 (BioLegend), followed by goat anti-mouse-IgG-horseradish peroxidase Ab (Dako, Agilent Technologies, Santa Clara, Calif). Rabbit anti-human anti-H3 mAb (Abcam, Cambridge, UK) was used as control for loading, followed by rabbit anti-mouse-IgG-horseradish peroxidase Ab (Dako).

Statistical analyses

To evaluate the correlation between the frequency of FOXP3-expressing cells and the plasma levels of rapamycin, the nonparametric Spearman's rank correlation coefficient was used. $P < .05$ was considered significant.

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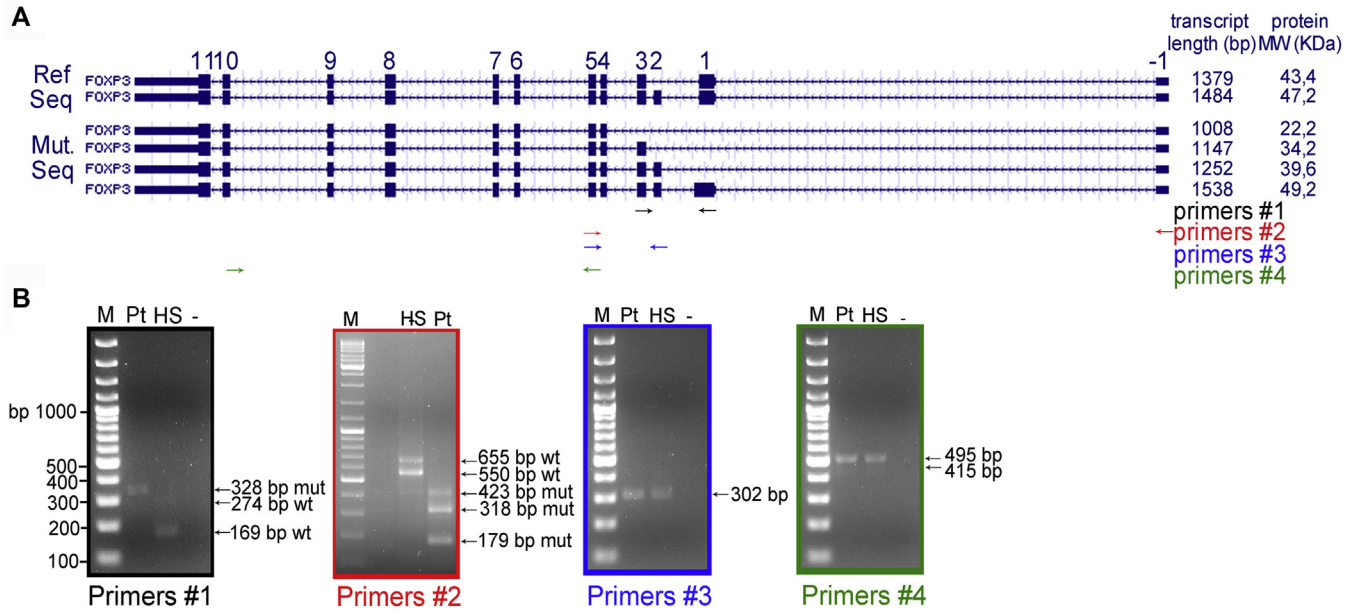

FIG E1. *FOXP3*-mutation c.210+1G>C results in aberrant mRNA splicing isoforms. **A,** Reference (*Ref*) sequences (*Seqs*) of wt*FOXP3* encoding mRNA (UCSC Genome Browser, https://genome.ucsc.edu/cgi-bin/hgGateway; University of California—Santa Cruz Genomics Institute, Santa Cruz, Calif) and reconstruction of the aberrant splicing isoforms detected in RNA from activated PBMCs of patient 24, carrying the genomic mutation (*Mut*) c.210+1G>C. Position of primers used for amplification of the different isoforms are reported as arrows at the bottom. Transcripts length and predicted molecular weight of the corresponding proteins are reported in the *left* columns. **B,** Primers reported in **A** were used to amplify cDNA from activated PBMCs of patient 24 and an HS; relative PCR products are shown. Single amplicons were subjected to Sanger sequencing, reconstruction of the corresponding transcripts detected in patient 24 is reported in **B**. Mutated isoform 4 is likely to be expressed at extremely low level, because it can be detected only when specific primers are used (primer 1). Numbers on the *right* indicate the length of the amplicons. *M*, Molecular weight marker.

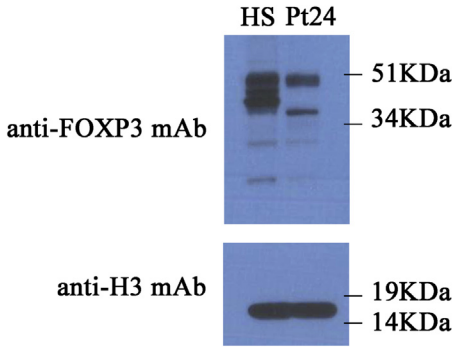

FIG E2. Aberrant FOXP3 isoform expression in the PB of patient 24, who has IPEX syndrome. PBMCs from HSs or patient 24 were activated for 72 hours in the presence of anti-CD3 and anti-CD28 mAbs, prior to cell lysis and protein extraction. A 10-μg sample of total proteins was loaded per lane of a polyacrylamide gel. After transfer onto nitrocellulose filters, membranes were probed with mouse anti-human FOXP3 mAb (*upper*) and anti-human H3 mAb (*lower*). Molecular weight was determined based on prestained molecular weight marker run in parallel.

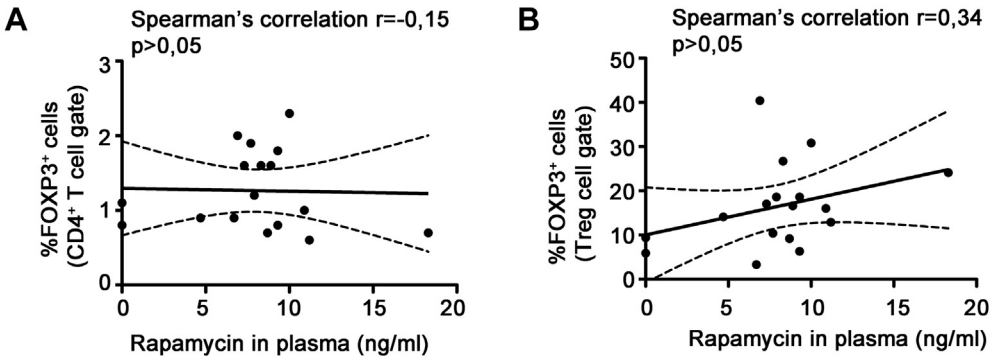

FIG E3. FOXP3 expression in peripheral $CD4^+$ T cells does not significantly correlate with rapamycin plasma levels. The correlation between the levels of rapamycin measured in the serum of patient 24 and the frequency of FOXP3-expressing cells in the $CD4^+$ T-cell compartment (**A**) or in the $CD4^+CD25^+CD127^{low}$ Treg subset (**B**) measured by flow cytometry was tested by Spearman rank correlation coefficient (n = 17 independent measurements). Correlation coefficients are indicated in the graphs.

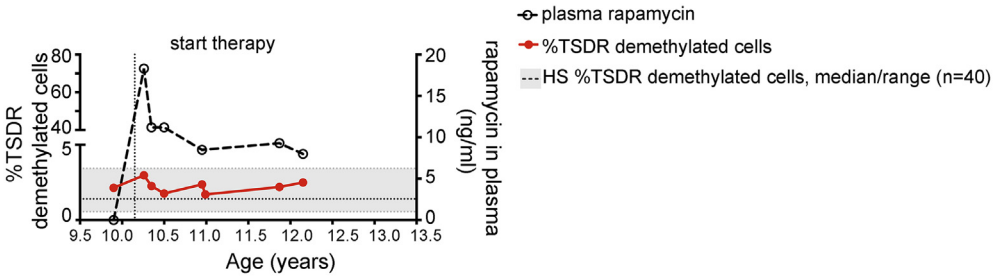

FIG E4. Constant frequency of circulating TSDR-demethylated tTreg cells during rapamycin therapy. The frequency of tTreg cells in PB was measured by molecular TSDR assay and is reported as percentage over total nucleated cells in PB. Dark lines in all graphs depict rapamycin plasma levels measured at different time points. Shaded areas indicate reference values in HS[E1] (median [range], n = 40).

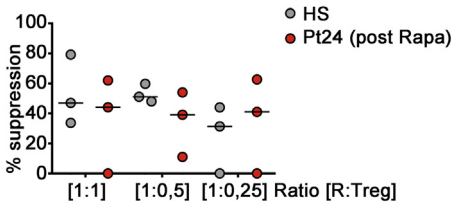

FIG E5. Rapamycin-conditioned IPEX Treg cells display *in vitro* suppressive function versus autologous responder cells. The suppressive ability of patient 24's Treg cells was tested several times during rapamycin therapy at different R to Treg cell ratios using patient's $CD4^{+}CD25^{-}$ Teff cells as Rs. The %suppression medians (ranges) were Pt ratio 1:1: 44.1 (0.0%-62.0%), n = 3; 1:0.5: 39.1 (11.0%-54.0%), n = 3; 1:0.25: 41.0 (0.0%-44.0%), n = 3; and HS ratio 1:1: 47.0 (33.7%-79.2%), n = 3; 1:0.5: 51.1 (48.0%-59.8%), n = 3; 1:0.25: 31.4 (0.0%-44.0%), n = 3. Relative %suppression is plotted in the graphs, lines indicate median.

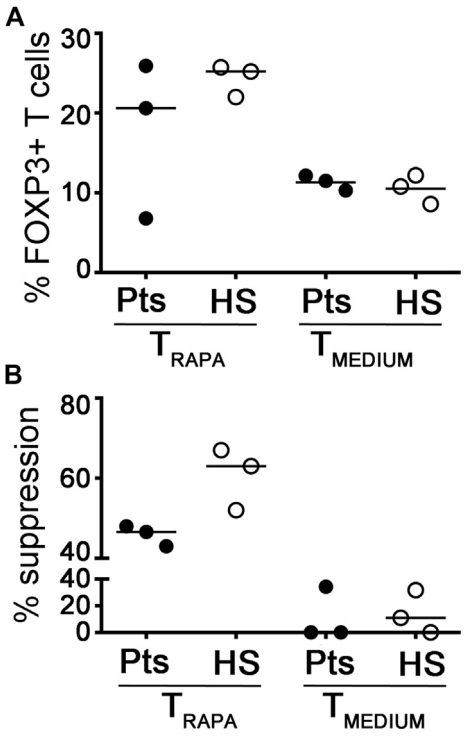

FIG E6. IPEX CD4$^+$ T cells expanded *in vitro* in the presence of rapamycin acquire suppressive ability. Purified CD4$^+$ T cells were expanded *in vitro* in the presence of rapamycin 100 nmol/L. After 3 rounds of stimulation, (**A**) intracellular FOXP3 staining (%FOXP3$^+$ T cells median [range]: HS T_{rapa} 25.2% [22.0%-25.74%]; HS T_{medium} 10.8% [8.6%-12.2%], n = 3; Pt T_{rapa} 20.6% [6.8%-25.9%]; Pt T_{medium} 11.5% [10.3%-12.1%], n = 3) and (**B**) *in vitro* suppression assay using HS PBMCs as Rs at R to suppressor cells ratio of 1:1 were performed (%inhibition of CD4$^+$ T cell proliferation median [range] was 63.0% [52.0%-67.0] at R to HS T_{rapa} = 1:1; 46.6% [43.0%-48.05] at R to Pt T_{rapa} = 1:1, n = 3). Lines in the graphs indicate median values. Clinical and molecular details of patient 20 were previously described;[E2,E3] patient 26 is an adult patient with IPEX syndrome (*FOXP3* mutation R347H) devoid of IS therapy at time of sampling.

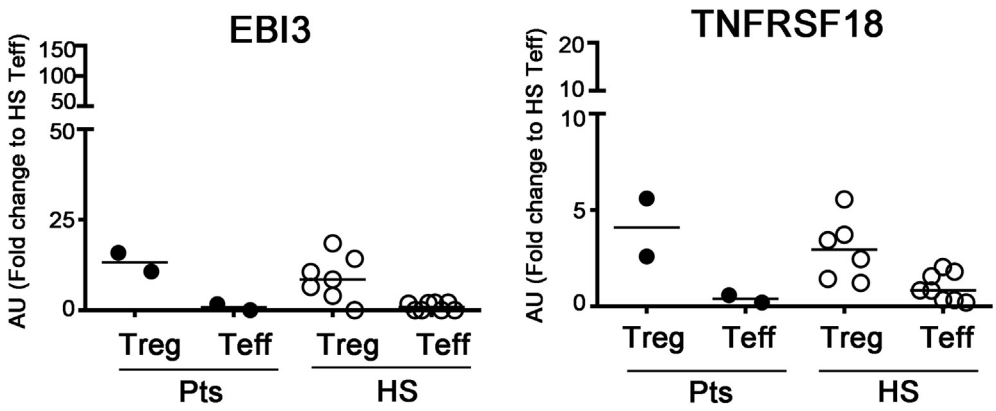

FIG E7. Normal expression of Ebi3 and GITR mRNA in Treg cells from patients with IPEX syndrome who are devoid of rapamycin treatment. The expression of EBI3 and TNFRSF18 mRNA was detected by ddPCR using RNA extracted from $CD4^+CD25^+CD127^{low}$ Treg cells and $CD4^+CD25^-$ Teff FACS-sorted from PB of 2 patients with IPEX syndrome who were not under rapamycin treatment at the time of analysis (patient 29, sibling of patient 28, with no clinical manifestations of the disease and devoid of therapy at the time of analysis,- and patient 30) (Table E1) and HSs (n = 7). Expression (detected as target transcript copies/μL of PCR reaction) was normalized to the expression of the housekeeping gene *PGK* and plotted as fold change to the average expression detected in HS Teff.

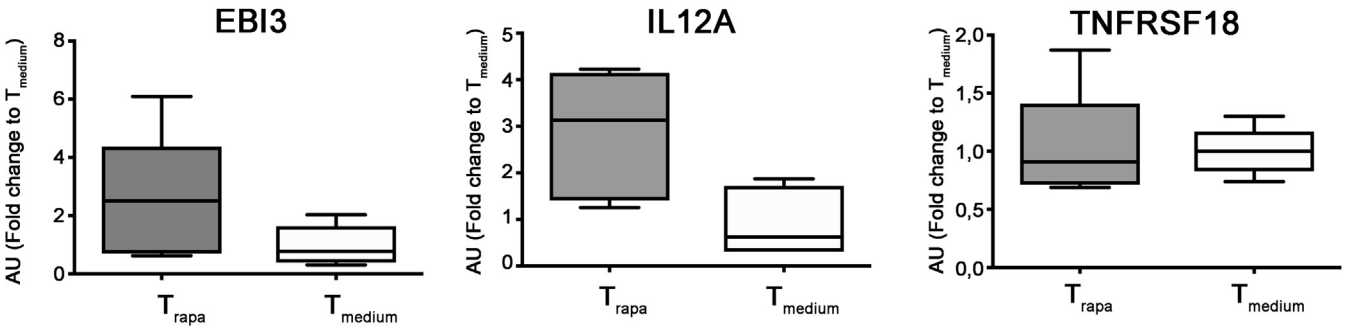

FIG E8. T_{rapa} cells from HS upregulate IL-12A and Ebi3 expression. Ebi3, IL-12A (p35), and TNFRSF18 mRNA expression in HS $CD4^+$ T cells after 3 rounds of *in vitro* stimulation in the presence (gray bars, T_{rapa} cells) or absence (white bars, T_{medium} cells) of rapamycin is plotted in the graphs. Expression of the target genes was normalized to the expression of the housekeeping gene PGK and plotted as fold change to the average expression detected in T_{medium} cells, arbitrarily set as 1. AU median (range): Ebi3 T_{rapa} 2.5 (0.6-6.1) versus T_{medium} 0.8 (0.3-2.0) fold change to average T_{medium} cells; IL-12A (p35): T_{rapa} 3.1 (1.3-4.2) versus T_{medium} 0.6 (0.3-1.9$_)$; TNFRSF18 T_{rapa} 0.9 (0.7-1.9) versus T_{medium} 1.0 (0.7-1.3), n = 5, *P* = ns, nonparametric Mann-Whitney.

TABLE E1. Clinical features of patients with IPEX syndrome under rapamycin therapy

Patient	Mutations	FOXP3 protein expression	Ongoing therapy at sampling	Duration of therapy	Major autoimmune manifestations		IgE UI/mL	Lymphocytes/mm^3	Treg % of CD4	Eos/mmc	HAA/VAA
					Pre Rapa	On Rapa	On Rapa				
24	c.210+1G>C	No	Rapa	3 y	Gastritis, FTT	Solved gastritis	20.0	3600	4.9	800	Pos/neg
25	p.R347H (c.1040G>A)	Yes	CsA*	10 mo	Diarrhea, FTT, eczema	Solved diarrhea	0.4	1900	7.4	380	Pos/nt
			Rapa	7 mo							
27	p.V480M (c.1222G>A)	Yes	Rapa	16 mo	Diarrhea, FTT	Recurrence of chronic diarrhea	n.a.	3180	5.8	90	Pos/neg
			Predn	4 y							
			Anti-TNF-α	4 y							
28	p.R397Q (c.1190G>A)	Yes	Steroids*	11 mo	Diarrhea, thyroidism	Solved diarrhea	80.3	5800	7	200	Pos/neg
			Rapa	24 mo							
			Aza	8 mo							
30	c.736-1G>A	Yes	Rapa	2 mo	Diarrhea, FTT	Improved diarrhea	n.a.	2340	8.4	10	Pos/pos
31	p.R337Q (c.1010G>A)	Yes	Steroids	2 mo	Diarrhea, T1D, eczema, nephropathy	Improved diarrhea	n.a.	2725	6	408	Pos/neg
			Rapa	2 mo							
			Rituximab	4 doses							

Aza, Azathioprine; *CsA*, Cyclosporin A; *Eos*, eosinophils; *FTT*, failure to thrive; *HAA*, anti-harmonin autoantibodies; *n.a.*, not available; *neg*, negative; *n.t.*, not tested; *pos*, positive; *Predn*, prednisolone; *T1D*, type 1 diabetes; *UI*, International Units; *VAA*, anti-villin auto-antibodies.

Patients were treated with rapamycin alone or in combination with other IS agents.

Clinical and immunologic data refer to the indicated therapy duration.

*Tapering ongoing.

TABLE E2. Treg phenotype in PB of patients with IPEX syndrome who are under rapamycin treatment

Markers (gating strategy)	Pts IPEX (n = 5) %positive median (range)	HSs (n = 16-27) %positive median (range)	*P* value
$CD25^{+}CD127^{low}$ ($CD4^{+}$)	7.0 (5.8-8.4)	5.2 (1.8-9.4)	Ns*
$FOXP3^{+}$ ($CD4^{+}$)	9.0 (2.3-16.8)	6.0 (1.8-10.8)	Ns*
$FOXP3^{+}$ ($CD4^{+}CD25^{+}CD127^{low}$)	67.3 (27.2-82.6)	74.5 (42.6-91.6)	Ns*
$CD25^{+}$ ($CD4^{+}$)	15.3 (13.1-17.5)	28.2 (11.3-37.0)	.01
$CD39^{+}$ ($CD4^{+}$)	15.7 (10.6-19.3)	6.6 (1.8-10.6)	<.0001

Frequency of the indicated T-cell population was measured by flow cytometry in PB of patients with IPEX syndrome during rapamycin therapy and of HSs. Data are reported as percentage of marker-positive cells, median (range), in the indicated cell gate.
*Nonparametric Mann-Whitney.

TABLE E3. Gene expression analysis—fold change to HS Teff cells

Gene target	IPEX Treg (n = 6)	HS Treg cells (n = 7)	IPEX Teff (n = 6)	HS Teff (n = 8)
FOXP3	101.1 (56.9-182.7)	115.0 (73.0-180.5)	4.2 (1.6-36.1)	0.7 (0.3-2.9)
LRRC32	42.4 (24.7-123.7)	82.7 (49.0-111.9)	0.6 (0.0-2.8)	0.8 (0.2-3.1)
TGFB1	1.1 (0.8-1.3)	0.9 (0.8-1.4)	0.9 (0.7-1.2)	1.0 (0.6-1.5)
IL-12A	1.8 (0.6-5.2)	2.2 (1.5-4.6)	0.9 (0.5-1.2)	0.9 (0.5-1.7)
EBI3	52.9 (14.6-105.8)	8.5 (0.0-18.5)	6.4 (2.4-126.3)	0.9 (0.0-2.1)
TNFRSF18	7.1 (5.4-15.8)	3.0 (1.2-5.6)	0.6 (0.2-5.6)	0.8 (0.2-2.1)
IL-10	9.5 (5.2-28.4)	9.5 (3.8-49.0)	0.8 (0.0-1.7)	0.8 (0.2-1.8)

ddPCR analysis of gene expression on sorted peripheral $CD4^+CD25^+CD127^{low}$ Treg cells $CD4^+CD25^-CD127^+$ Teff cells from patients with IPEX syndrome with ongoing rapamycin therapy and from HSs. Data are expressed as AU (fold change to the average expression in HS Teff cells). Median values (range) of each target gene are listed.

TABLE E4. Gene expression analysis—relative expression

Gene target	IPEX Treg	HS Treg cells	IPEX Teff	HS Teff
FOXP3	1.02 (0.57-1.85)	1.28 (0.74-1.82)	0.04 (0.02-0.36)	0.01 (0.00-0.03)
LRRC32	0.048 (0.028-0.142)	0.095 (0.056-0.128)	0.001 (0.000-0.003)	0.001 (0.000-0.004)
TGFB1	2.49 (1.98-3.09)	2.20 (1.92-3.40)	2.22 (1.60-2.86)	2.38 (1.51-3.45)
IL-12A	0.015 (0.006-0.028)	0022 (0.015-0.047)	0.008 (0.005-0.012)	0.009 (0.006-0.017)
EBI3	0.0031 (0.0009-0.0062)	0.0005 (0.0000-0.0011)	0.0004 (0.0001-0.0074)	0.0001 (0.0000-0.0001)
TNFRSF18	0.097 (0.073-0.214)	0.040 (0.017-0.075)	0.008 (0.003-0.076)	0.011 (0.003-0.028)
IL-10	0.009 (0.005-0.027)	0.009 (0.004-2.953)	0.001 (0.000-0.002)	0.001 (0.000-0.002)

ddPCR analysis of gene expression on sorted peripheral $CD4^+CD25^+CD127^{low}$ Treg cells and $CD4^+CD25^-$ Teff cells from patients with IPEX syndrome with ongoing rapamycin therapy and from HSs. Data are expressed as number of copies/μL of the test gene normalized to number of copies/μL of *PGK*, used as housekeeping gene for normalization of samples. Median (range) expression of each target gene are listed.