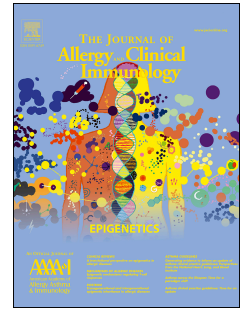


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Treatment with Rapamycin Can Restore Regulatory T Cell Function in IPEX Patients

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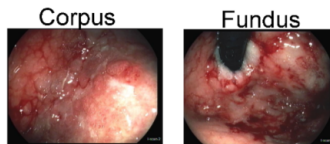
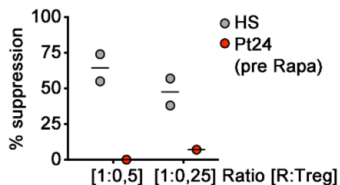
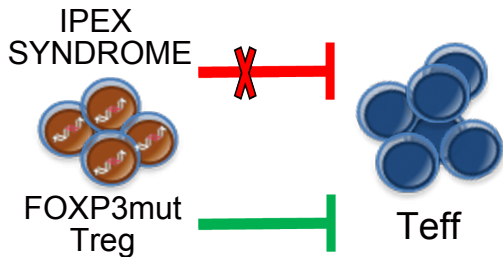
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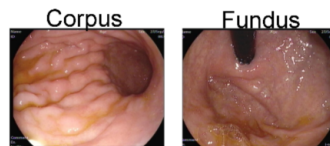
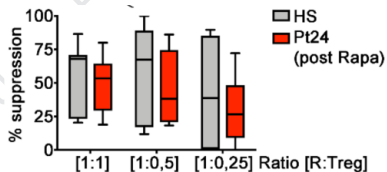
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Treatment with Rapamycin Can Restore Regulatory T Cell Function in IPEX Patients



PRIOR TO RAPAMYCIN THERAPY



ON RAPAMYCIN THERAPY

Abbreviations:

IPEX: Immune-dysregulation, polyendocrinopathy, enteropathy, X-linked;
 FOXP3: Forkhead-box-P3; mut: mutated
 Treg: regulatory T cells; Teff: T effector cells; R: responder cells;
 HS: Healthy subject; Pt: patient;
 Ebi3: Epstein-Barr-virus-Induced-3;
 GITR: glucocorticoid-induced-TNFR-related.

Restored Treg function,
 increased Treg stability
 via FOXP3-independent mechanism
 (by increasing EB13 and TNFRSF18)

Treatment with Rapamycin Can Restore Regulatory T Cell Function in

IPEX Patients.

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Abstract.

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 (t) regulatory T cells (Tregs), i.e., Forkhead-box-P3 (*FOXP3*), resulting in impaired Treg function and autoimmunity. At present, hematopoietic stem cell transplantation is the therapy of choice for IPEX patients. 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 Tregs expressing wild-type *FOXP3*, thereby proving beneficial in the clinical setting of immune-dysregulation. However, the mechanisms of immunosuppression selective to IPEX patients' Tregs are unclear.

Objective: To determine the cellular and molecular basis of the clinical benefit observed under rapamycin treatment in six IPEX patients with different *FOXP3* mutations.

Methods: We tested phenotype and function of *FOXP3*-mutated Tregs from rapamycin-treated IPEX patients by flow cytometry and *in vitro* suppression assays, and the gene expression profile of rapamycin-conditioned Tregs by droplet-digital-PCR.

Results: Clinical and histological improvements in patients correlated with partially restored Treg function, independent of *FOXP3* expression or Treg frequency. Expression of Tumor-Necrosis-Factor-Receptor-Superfamily-Member18 (*TNFRSF18*, GITR) and Epstein-Barr-virus-Induced3 (*EBI3*, an IL-35 subunit) in patients' Tregs increased during treatment as compared to that of Tregs from untreated healthy subjects. Furthermore inhibition of GITR and Ebi3 partially reverted *in vitro* suppression by *in vivo* rapamycin-conditioned Tregs.

Conclusion: Rapamycin is able to impact on Treg suppressive function *via* a *FOXP3*-independent mechanism, thus sustaining the clinical improvement observed in IPEX patients under rapamycin treatment.

Clinical Implications. Rapamycin therapy allows clinical, histological and immunological improvement in patients with Immune-dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome, supporting its preferential use to overcome their Treg dysfunction.

Capsule Summary. Rapamycin administration restores the suppressive function of *FOXP3*-mutated regulatory T cells *via* a *FOXP3*-independent mechanism, leading to significant and persistent clinical and histological improvement of autoimmunity in Immune-dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome.

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

Abbreviations.

- ddPCR: Droplet digital polymerase chain reaction
- Ebi3: Epstein-Barr-virus-Induced-3
- FOXP3: Forkhead-box-P3
- GITR: glucocorticoid-induced-TNFR-related
- HS: Healthy subject
- HPRT: Hypoxanthine-guanine phosphoribosyltransferase
- IL: interleukin
- IPEX: Immune-dysregulation, polyendocrinopathy, enteropathy, X-linked
- LRRC32: leucine-rich repeat containing protein 32
- LV: Lentiviral vector
- mAb: monoclonal antibody
- mTOR: mammalian target of rapamycin
- PB: peripheral blood
- PBMC: peripheral blood mononuclear cells
- PGK: Phosphoglycerate kinase
- Pt: Patient
- t: thymus-derived
- TCR: T-cell receptor
- Teff: Effector T
- TGF β : Transforming Growth Factor β
- TNFRSF18: Tumor-Necrosis-Factor-Receptor-Superfamily-Member18
- Treg: Regulatory T cell
- TSDR: Regulatory T cell-specific demethylated region
- wt: wild-type

Introduction

Forkhead-box-P3 (FOXP3) is a transcription factor critical for the function of thymus-derived (t) regulatory T cells (Tregs), a subset of CD4⁺ T lymphocytes devoted to the maintenance of self-tolerance¹. *FOXP3* mutations result in impaired Treg function, the key pathogenic event leading to autoimmunity in Immune-dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome². Typically, 90% of patients manifest the disease within the first year of life³. The most common symptoms are enteropathy, skin disorders, type-1-diabetes (T1D), cytopenia, hepatitis, although milder manifestations, including kidney abnormalities and arthritis, have been reported³. Current treatments include supportive and immunosuppressive therapies, and allogeneic hematopoietic stem cell transplantation (HSCT)²⁻⁴. According to the largest reported IPEX patients cohort, HSCT 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-HSCT³. 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 autoimmunity³. Rapamycin has been reported to induce clinical remission in several patients with long-term follow-up and its use in IPEX 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 tTregs and enhances Foxp3 expression¹⁰. In humans, rapamycin spares and expands CD4⁺CD25⁺FOXP3⁺ Tregs *in vitro*, while inhibiting the proliferation of

131 effector T (Teff) cells¹¹. Rapamycin treatment fosters the function of Tregs in T1D patients¹²
132 and Treg expansion in kidney transplant recipients¹³. Clinical improvement upon rapamycin
133 treatment has also been reported in patients with primary immunodeficiencies with prevailing
134 lymphoproliferation, such as Autoimmune lymphoproliferative syndrome (ALPS), Activated
135 phosphoinositide 3-kinase d syndrome, LPS-responsive beige-like anchor protein
136 (LRBA) deficiency, and CTLA4 insufficiency¹⁴⁻¹⁶. However, to date, the beneficial effect of
137 rapamycin has only been reported for Tregs expressing wild-type (wt)*FOXP3*.
138 Here, we have investigated the cellular and molecular impact of rapamycin treatment in IPEX
139 patient Tregs, to determine if rapamycin increases the number and/or function of *FOXP3*-
140 mutated Tregs, and if this increase is *via* *FOXP3*-dependent or -independent mechanisms.
141 Results indicate that the clinical improvement is associated with partial restoration of Treg
142 function without increase of *FOXP3* expression. We propose that rapamycin increases the
143 stability of *FOXP3*-mutated Tregs, likely by restoring mTORC inhibition lost due to *FOXP3*
144 mutations, and by modulating Treg-specific pathways, such as those mediated by GITR and
145 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 upon informed consent, in accordance with the OSR Ethical Committee (TIGET06/Periblood). IPEX patients recruited under TIGET06 protocol follow internal OSR numbering, previously applied to earlier publications¹⁷⁻²¹. 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 2-3 mg/mq on day 1, followed by a maintenance dose of 1mg/mq/day, than adjusted to target the plasmatic level to 8-12 ng/mL, given the previous experience in IPEX patients⁵⁻⁷.

In Pt24, starting from 2 mg/mq, 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, peripheral edema, and were periodically monitored as appropriate. No side effects were observed in the present cohort except for Pt24 who developed severe oral aphthous lesions which 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. 3µm sections were stained by routine haematoxylin/eosin by standard histological techniques.

Cell culture

Biopsies were processed as described²² and cell suspensions were activated *in vitro* in the presence of plate-bound anti-CD3 10µg/ml (Miltenyi-Biotec, Bergisch Gladbach, Germany), soluble anti-CD28 1µg/ml (BD, Franklin Lakes, NJ) mAbs and rhIL-2 100U/ml (Proleukin, Novartis, Basel, Switzerland). After 10-day expansion, T-cell lines were analysed by flow-cytometry.

PB-mononuclear-cells (PBMC) were purified over Ficoll-Hypaque gradients. For suppression assays and mRNA extraction CD4⁺CD25⁺CD127^{low} (Tregs) and CD4⁺CD25⁻ (Teff) cells were FACS-sorted (MoFloXDP, Beckman-Coulter, Brea, CA). All samples were over 95% pure.

For 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 10µg/ml (Miltenyi-Biotec) and anti-CD28 1µg/ml (BD)mAbs in the presence or absence of 100nM Rapamune (Whyet, Madison, NJ) for three-rounds of stimulation¹¹. All cultures were performed in X-VIVO15/5%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 5hours with Phorbol-12-myristate-13-acetate (PMA) 10ng/ml plus ionomycin 1µg/ml in the presence of Brefeldin-A 10µg/ml (all from Sigma-Aldrich, Saint Louis, MO).

Flow-cytometry

Anti-CD4, anti-CD25, anti-CD62L mAbs (all from BD), anti-TIGIT (eBioscience, San Diego, CA), anti-CD39, anti-CD127, anti-CD95 and anti-CD4 mAbs (all from Biolegend, San

Diego, CA) were used to prepare antibody cocktails for surface staining. Intracellular staining with anti-CTLA4, anti-FOXP3, anti-Helios (all from Biolegend), anti-Ki67 (BD) mAbs was performed after fixation/permeabilization with FOXP3-staining-kit (eBioscience). Intracellular-cytokine staining was performed after fixation (2%Formaldehyde, Thermo-Fisher-Scientific, Waltham, MA), and permeabilization (0,5%Saponin, Sigma-Aldrich), using an antibody cocktail containing anti-human-IFN γ (BD), anti-IL-2, anti-IL17A, and anti-IL-4 mAbs (eBioscience). Samples were acquired on BD-FACSCantoII and analyzed with FCS-Express-Pro-Software-4 (De Novo, Glendale, CA) or FlowJo (BD).

Nucleic acid extraction and polymerase-chain-reaction

RNA was extracted with RNeasy-kits (Qiagen, Hilden, Germany) and reverse-transcribed with High-Capacity cDNA Reverse-Transcription-Kit (Applied-Biosystems, Foster City, CA). Gene expression was assessed on 20ng of RNA-equivalent by droplet-digital-polymerase-chain-reaction (ddPCR) (QX200, Bio-Rad, Hercules, CA) 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, interleukin-10 (IL-10), Tumor-Necrosis-Factor-Receptor-Superfamily-Member-18 (TNFRSF18), Epstein-Barr-virus-Induced-3 (Ebi3), and IL12A mRNAs were quantified with Assay-on-Demand RT-PCR kits (Applied-Biosystems). Relative expression is plotted as Arbitrary-Units (AU: 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, CA). T cells were activated at a density of 20000cells/well with anti-CD3/CD28/CD2-coated beads (Milteny-Biotec) at [bead:T-cell] ratio [1:1]. FACS-sorted Tregs were added at [R:Tregs] ratio of [1:1]/[1:0,5]/[1:0,25]. After a 6-day co-culture the

percentage of divided precursors was assessed by flow-cytometry. Suppression was calculated by comparing %divided cells in the absence or presence of Tregs. When indicated, the following mAbs were added: 10 μ g/ml anti-human-TGF β 1,2,3, anti-human-GITR, and anti-human-IL12A mAbs, isotype control (R&D-Systems, Minneapolis, MI), or anti-human-Ebi3 mAb (Millipore, Burlington, MA).

Statistical Analyses

Results are presented as median/range or mean $\pm$ SEM, as indicated. Analyses were performed with non-parametric tests (Mann-Whitney/Wilcoxon). $p < 0.05$ was considered significant. Statistical analysis was applied to groups of five or more independent results.

Results

1: Clinical and immunological evolution of IPEX pathology in response to rapamycin

treatment. We report the clinical follow-up of an IPEX Patient (Pt24) who received a 3-year-treatment with rapamycin monotherapy with clinical remission of the disease. Pt24 was 9 years-old when he was referred to our clinic for severe 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 morphological or immunohistochemical signs of *Helicobacter Piloni* infection or autoimmune gastritis. Mild duodenal villous hypotrophy and increased intraepithelial lymphocytes were observed, but minimal involvement of colon (Fig.1A,B). A dysimmune origin was suggested. Suspicion of IPEX syndrome was raised based on high titer of anti-harmonin-autoantibodies

(HAA>100U)²⁰ and low FOXP3 expression within the peripheral CD4⁺CD25⁺CD127^{low} T

cells (Fig.1C). *FOXP3* sequencing revealed a mutation at exon+1 splice-junction

(c.210+1G>C)³, resulting in aberrant mRNA splicing isoforms (Fig.E1) and aberrant protein

expression (Fig.E2). We started rapamycin treatment (target plasma level: 8-12µg/ml);

steroids were added to treat aphtosis at occurrence. Upon 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.1D). Lymphocytes infiltrating the gut mucosa

were isolated and characterized prior and during therapy (Fig.2A,B). The frequency of Tregs

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.2A), while the frequency of T

effector cells, as determined by cytokine production after short-term *in vitro* expansion of

infiltrating T cells, drastically decreased overtime, except for residual Th17 cells in the *fundus*

(Fig.2B). The regulatory-skew of the T-cell infiltrate (Fig.2A) correlated with progressive

histological reduction of the mucosal inflammation (Fig1D). Stable recovery of tissue

architecture was achieved at 26 months of treatment, although residual patches of hypotrophic mucosa were present (not shown).

2: Rapamycin treatment does not result in increased frequency of circulating FOXP3-mutated Tregs. We monitored the frequency of Tregs in the PB of Pt24 after initiation of rapamycin treatment (Fig.3). Unlike that observed in the gut tissue, the frequency of peripheral FOXP3⁺ T cells remained low upon treatment (Fig.3A), and there was no correlation in increased FOXP3 expression with rapamycin plasma levels (Spearman's correlation $p > 0.05$, Fig.E3). Accordingly, the sporadic peaks of FOXP3-expressing T cells were not due to expansion of tTregs, as demonstrated by constant frequency of TSDR-demethylated cells in PB (Fig.E4). Further, *in vivo* Treg proliferation, measured by the proliferation-marker Ki67, was not increased (Fig.3B). The expression of Treg-associated markers, including Helios, CTLA4, TIGIT, CD95, CD62L, was comparable to that detected in HS Tregs (Fig.3B), with the exception of CD39 ($p = 0.0004$, HS $n = 10$ vs Pt24 $n = 9$, Mann-Whitney, Fig.3C) and CD25, whose expression was low during therapy ($p < 0.0001$, HS vs Pt24 $n = 18$, Mann-Whitney, Fig.3C). Overall, these data indicate that rapamycin resulted in enriched Tregs in the target tissue, but did not increase the frequency of peripheral FOXP3-mutated Tregs, nor induced mutant FOXP3 expression.

3: Rapamycin treatment restores the suppressive function of FOXP3-mutated Tregs.

At diagnosis and before any treatment, the CD4⁺CD25⁺CD127^{low} Tregs of Pt24 did not show suppressive function *in vitro* (Fig.3D). The assay was repeated in 9 independent assays from 2 months to 3 years after initiation of rapamycin therapy at different [responder:Treg] 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 Tregs, rapamycin stably restored Treg function (Fig.3E). Data were also confirmed when Pt24 CD4⁺CD25⁻ T cells were used as responders (Fig.E5).

We further analysed Tregs from 5 IPEX patients under rapamycin treatment (TableE1/Fig.4). Unlike Pt24, for all other patients rapamycin was not the only IS treatment, but rapamycin was administered while tapering alternative IS drugs (TableE1 for concomitant medications at time of sampling). Although only tested during treatment, the frequency of peripheral Tregs, 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.4A-B/TableE2), likely depending on FOXP3 mutation site (Table E1). Similar to findings in Pt24 PBMC (Fig.3C), expression of CD25 in CD4⁺ T cells was lower in patients under therapy than in HS (*: $p<0,05$, Mann-Whitney; Fig.4C/TableE2), likely due to reduced frequency of activated CD4⁺ T cells. In contrast, CD39, whose expression remained impaired in Pt24 (Fig.3C), was higher in this cohort of patients than in HS (**: $p<0,01$, Mann-Whitney; Fig.4C/TableE2), in line with the known effect of rapamycin on CD39²³. We tested the suppressive ability of Tregs from 4 out of 5 treated IPEX patients (Pt25/28/30/31) at a [Responder:Treg] ratio of [1:1] and [1:0,5] (Fig.4D). We found that some level of suppressive activity was detectable in most patients, although their function was significantly lower than that detected in HS ($p=0,02$, Wilcoxon paired-test, Fig.4D). These results indicate that *FOXP3*-mutated Tregs exposed to rapamycin *in vivo*, display suppressive activity *in vitro*, which might sustain the clinical improvement observed in these patients.

4: Rapamycin expands functional *FOXP3*-mutated Tregs *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 Tregs¹¹. To test this effect with mutant FOXP3, CD4⁺ T cells from 3 IPEX patients who were not under

rapamycin treatment at time of sampling (Pt20^{19,20}/24/26) were cultured in the presence of rapamycin. After *in vitro* expansion, as expected, the control HS T cells (hereafter T_{rapa}) were enriched for FOXP3-expressing cells, and displayed efficient suppression, whereas those expanded without rapamycin (T_{medium}) did not (Fig.E6A-B). In two (Pt20/26) out of the 3 IPEX patients tested, FOXP3-expressing cells were increased in T_{rapa} cultures (Fig.E6A). T_{rapa} from Pt24, with the splice-site mutation that prevents FOXP3 expression (Fig.1,E1), did not upregulate FOXP3-expressing cells (Fig.E6A). However, T_{rapa} of all the patients acquired suppressive function, although less potent than that observed by wtFOXP3 T_{rapa}, in line with results from *ex vivo* Tregs (Fig.E6B). These data suggest that the acquisition of suppressive function by Tregs after expansion in the presence of rapamycin is independent of the mutated FOXP3 residual expression.

5: Rapamycin upregulates Ebi3 and GITR expression in FOXP3-mutated Tregs.

To test the hypothesis that rapamycin-mediated restoration of the suppressive function in FOXP3-mutated Tregs, is *via* a direct impact on key Treg-associated molecules²⁴, we analyzed the expression of various genes by ddPCR using FACS-sorted CD4⁺CD25⁺CD127^{low} Tregs and CD4⁺CD25⁻ Teff cells from IPEX patients under rapamycin treatment (n=6 samples from 4 patients: Pts24/27/28/31) and HS (n=7) (Fig.5A/TableE3-E4). FOXP3 mRNA was highly expressed by IPEX Tregs, as in HS Tregs. As expected, LRRC32 (Glycoprotein-A-Repetition-Predominant (GARP)) was preferentially expressed by Tregs, where it contributes to Treg-mediated suppression by binding latent-TGFβ on activated Tregs²⁴, with no alterations in patients Tregs (Tregs-vs-Teff: p<0,0) (Fig.5A/TableE3-E4). TGFβ1 mRNA was expressed at comparable levels in both Tregs and Teff cells from HS and patients. In addition, IL-10 mRNA was preferentially expressed by Tregs both in patients and HS (Fig.5A/TableE3-E4).

The mRNAs for 2 proteins, i.e. Glucocorticoid-Induced-TNFR-Related-Protein (GITR) and Ebi3, showed significant increases when comparing HS and IPEX patient Tregs. While HS Tregs expressed 3-fold higher Glucocorticoid-Induced-TNFR-Related-Protein (GITR) mRNA (TNFRSF18, considered a marker of active Tregs²⁴) than Teff cells ($p<0,05$), the rapamycin-conditioned IPEX Tregs displayed significant up-regulation of GITR mRNA vs HS Tregs ($p<0,01$), suggesting contribution of this molecule to Treg-suppression during treatment. In addition, the two subunits of the immunosuppressive cytokine IL-35, i.e., Ebi3 and p35/IL12A, were also analyzed. Expression of IL12A was equally enriched in Tregs from both patients and HS. In contrast, Ebi3 mRNA was significantly higher in both Treg and Teff-derived IPEX samples, as compared to HS, with a 6-fold increase in rapamycin-conditioned vs HS Tregs ($p<0,01$ Mann-Whitney; Fig.5A/TableE3). However, the overall expression of IL-35 subunits was low in all samples tested (TableE4). Of note, when Ebi3 and GITR mRNA expression was measured in Tregs and Teff cells from 2 IPEX patients who were not under rapamycin therapy (Pts29/30), expression was comparable to that observed in HS (Fig.E8). In summary, expression of Ebi3 and GITR (TNFRSF18) increases during rapamycin treatment in IPEX patients' Tregs, which may contribute to the partial recovery of Treg-function.

We further tested the expression of IL-35 subunits and GITR mRNA in $T_{\text{rapa/medium}}^{11}$ from HS ($n=5$). 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} (Fig.E7). Therefore, while IL-35 subunits were upregulated upon rapamycin treatment in both *FOXP3*-mutated and wt T cells, GITR upregulation was a specific effect of *in vivo* treatment on IPEX Tregs.

6: Blockade of Ebi3/p35 and GITR reverts the suppressive activity of rapamycin-conditioned FOXP3-mutated Tregs.

We tested the effect of GITR and IL-35 blockade during *in vitro* suppression assays using multiple Treg samples from Pt24/Pt28 (Fig.5B). At [R:Treg] ratio of [1:1] no significant alteration of suppression was detected using both HS Tregs (n=4-6) and rapamycin-conditioned Tregs from patients (n=3-5 independent experiments), except for a non-significant reduction of the suppressive ability of both HS and Pt Tregs in the presence of anti-Ebi3/IL-12A mAbs ($p>0,05$, Wilcoxon paired test). At [R:Treg] ratio of [1:0,5] the suppression of HS Tregs 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 Tregs from IPEX patients 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 function¹⁰. These data suggest that, among the Treg-associated molecules we tested, Ebi3/IL-12A and GITR mainly contributed to improve suppressive ability of FOXP3-mutated Tregs during rapamycin treatment.

Discussion

We investigated the effects of rapamycin on Teff 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 one 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 favour of a regulatory-like T-cell infiltrate, a finding that correlated with tissue healing and clinical improvement. Concomitantly, *FOXP3*-mutated Tregs 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 Tregs. 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 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 cells²⁵. Accordingly, we report inhibition of mucosal T cells from gastric biopsies of an IPEX patient 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 expression²⁶. We speculate that Ebi3 up-regulation may contribute to both the dampening of Teff cells, including Th17 cells in the gut tissue, and the increase in tissue Tregs in the target organ.

Unlike other immunosuppressants, rapamycin can promote Treg expansion^{11,27}, although the underlying mechanism is not fully defined. This pro-tolerogenic 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 Tregs is dependent on FOXP3 *via* the Smgs1-PP2A pathway²⁸. Thus, *FOXP3* mutations may re-activate mTORC1 in Tregs with consequent decreased suppressive function, as reported in PP2A knock-out²⁸. In line with our results, recently published work by the group of T.A. Chatila demonstrates that *Foxp3* deficiency dysregulates mTORC2 signaling and metabolic pathways in Treg cells and that mTOR inhibitors restored suppressive function in *Foxp3*-deficient Tregs by enforcing a metabolic switch which suppresses the Teff-like reprogramming of dysfunctional Tregs²⁹. Although further studies are needed to dissect the underlying molecular mechanism, our data support the hypothesis that rapamycin re-establishes mTORC inhibition in *FOXP3*-mutated Tregs, 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 Tregs³⁰. The reported enhancing effect of rapamycin on CD39 expression in HS²³ was confirmed in all patients tested but Pt24, whose mutation abrogated full-length *FOXP3* expression, suggesting that restored Treg 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 Tregs. First, we find an increase in GITR expression, which leads to an activated or more stable Treg 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 Tregs³³. Later studies highlighted that IL-35 is associated with maximal Treg function or may mark highly suppressive human Tregs³⁴. Our results suggest that, although absolute Ebi3 mRNA levels were low, and no IL-35 cytokine was detected in PBMC supernatants (not shown), the up-regulation 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 Tregs. Based on the reported Ebi3-dependent induction of IL-17²⁶, rapamycin may improve the stability of Tregs by inhibiting the Th17-skew of unstable Tregs, a switch that has been observed when wtFOXP3 is absent¹⁹.

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

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Figure Legends

Figure 1. Altered gut histology and reduced FOXP3 expression in IPEX patient with

active disease. A, Images of Corpus/Fundus from endoscopy of Pt24 at diagnosis. **B,** Hematoxylin/eosin staining of gut biopsies of Pt24 at diagnosis. **C,** FOXP3 expression in PBMC of Pt24 at diagnosis (upper panels) and of a HS (lower panels). Left/middle panels: gate on $CD4^+$; right panels: gate on $CD4^+CD25^+CD127^{low}$. **D,** Images of Corpus/Fundus from endoscopy of Pt24 after 4 months therapy (right panels) and hematoxylin/eosin staining of gut biopsies of Pt24 on rapamycin therapy (26 months, left panels).

Figure 2. Decreased inflammatory infiltrates in IPEX during therapy with rapamycin.

Single-cell suspensions from biopsies (gastric mucosa) of Pt24 were activated with anti-CD3/CD28mAbs/rhIL-2 for 10 days. **A,** Right Y axis: frequency of Tregs (%FOXP3⁺ cells- dashed line, % $CD25^+CD127^{low}$ -grey line) within $CD4^+$; left Y axis: %FOXP3⁺ within $CD4^+CD25^+CD127^{low}$ Tregs (black filled line). **B,** Intracytoplasmic staining after stimulation with PMA/ionomycin/BrefeldinA. Black filled line: % $CD4^+/IFN\gamma^+IL-2^+IL17A^-IL-4^-$ (Th1), dashed line: % $CD4^+/IFN\gamma^+IL-4^+IL17A^-$ (Th2), grey line: $CD4^+/IFN\gamma^+IL17A^+IL-4^-$ (Th17).

Figure 3. FOXP3 expression in circulating Tregs during rapamycin therapy in IPEX

syndrome. A, Frequency of Tregs in PB of Pt24 before and during therapy: green line %FOXP3⁺; red line % $CD4^+CD25^+CD127^{low}$ (within $CD4^+$). Shaded areas: median values and range in HS (n=27). On X axis age of Pt24 is plotted. **B,** Expression of the Treg-associated markers Helios/CTLA4/TIGIT/CD95/CD62L and the proliferation-marker Ki67 was assessed at multiple timepoints during therapy (HS: n≥10; Pt24: n=2-16, gate $CD4^+CD25^+CD127^{low}$, median/range, p=ns). **C,** %CD39⁺ within $CD4^+CD25^+CD127^{low}$ and %CD25⁺ within $CD4^+$ prior and at multiple timepoints during therapy (HS: n≥10; Pt24: pre-therapy n=1; on-therapy n=18, median and range; ***:p<0,001, Mann-Whitney). **D,** %Suppression of Tregs from HS (n=2) and Pt24 pre-therapy ([R:Treg]=[1:0,5]: %suppression PtTregs: 0, n=1, HSTregs

572 64,5/55-74%-median/range n=2; [1:0,25]: %suppression Pt Tregs: 7, n=1; HSTregs 47,5/39-
 573 57% n=2). **E**, %Suppression of Tregs from HS and Pt24 on-therapy (median/range PtTregs
 574 [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;
 575 HSTregs [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-
 576 89,6%, n=6). p=ns, Mann-Whitney.

577 **Figure 4. Treg frequency and function in IPEX patients under rapamycin therapy. A**,
 578 %CD4⁺CD25⁺CD127^{low} and %FOXP3⁺ within CD4⁺ and **B**, %FOXP3⁺ within
 579 CD4⁺CD25⁺CD127^{low} in PB of patients on-therapy (n=5) and HS (n=27); p=ns, Mann-
 580 Whitney. **C**, %CD25/CD39 in the indicated cell-gate. *: p<0,05, **: p<0,01 vs HS (n=16),
 581 Mann-Whitney. **D**, %Suppression of Tregs from HS/Pts at [R:Treg] ratios of [1:1] or [1:0,5].
 582 Bars and errors indicate median and range; dots represent single assays. %suppression
 583 median/<range> at [1:1] ratio: Pts 34/<12-57>, HS 59/<33-98>, n=4; at [1:0,5] ratio: Pts
 584 0/<0-10>, HS 72/<52-100>, n=3; *: p<0,05, Wilcoxon paired-test.

585 **Figure 5. Rapamycin modulates IPEX Treg expression profile. A**. The expression of
 586 FOXP3/TGFB1/LRRC32/IL-10/TNFRSF18/EBI3/IL12A mRNAs by ddPCR on FACS-
 587 sorted Tregs and Teff (n=6 samples from n=4 patients–Pts24/27/28/31; HS n=7). **: p<0,01;
 588 *: p<0,05, Mann-Whitney. Arbitraty-Units: fold-change to average in HS Teff. **B**,
 589 %Suppression of Tregs from HS/Pts on-therapy in the presence of anti-TGF-β1,2,3 (black),
 590 antiEbi3/-IL12A (light-grey), anti-GITR (dark-grey) mAbs, or isotype-control (white).
 591 Mean±SEM: [R:Treg]=[1:0,5]: Isotype-control: 37,6±8,9%, n=5; anti-Ebi3/IL-12A:
 592 26,6±7,0%, n=4; anti-GITR: 18,9± 14,5%, n=3. HS n=4-6; Pt n=3-5 independent experiments
 593 on 2 patients on-therapy (Pt24/28).

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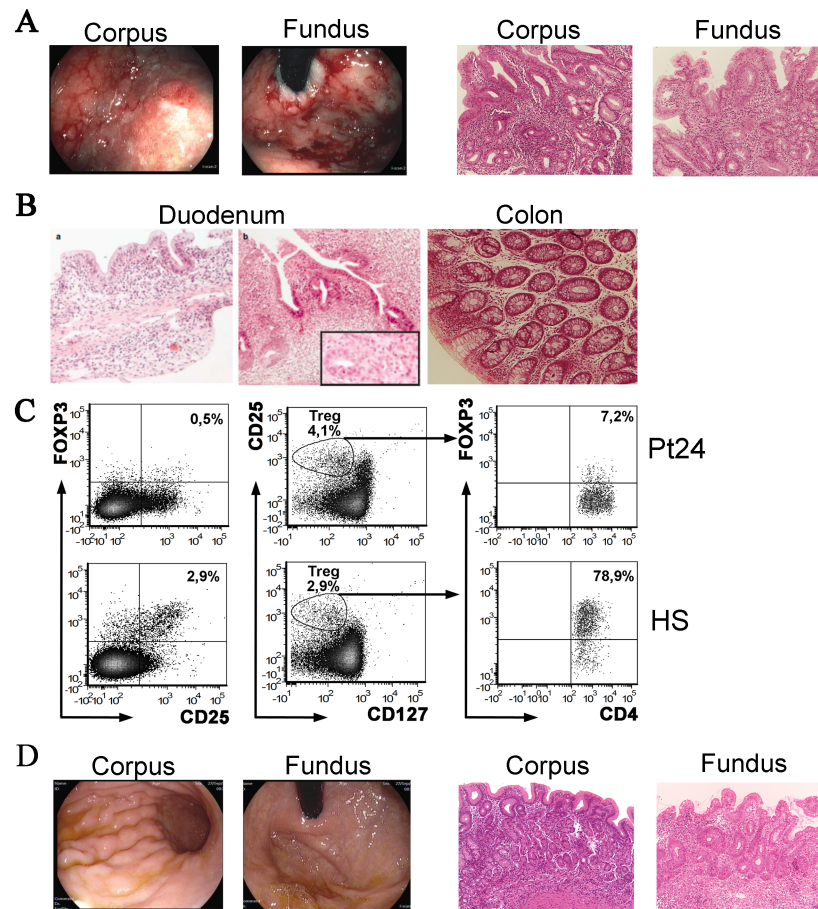


Figure 1

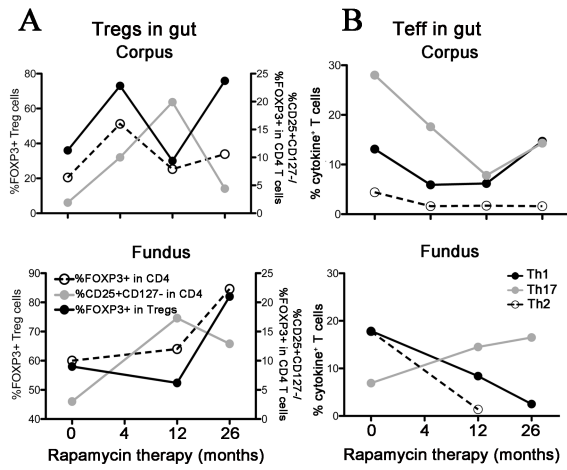


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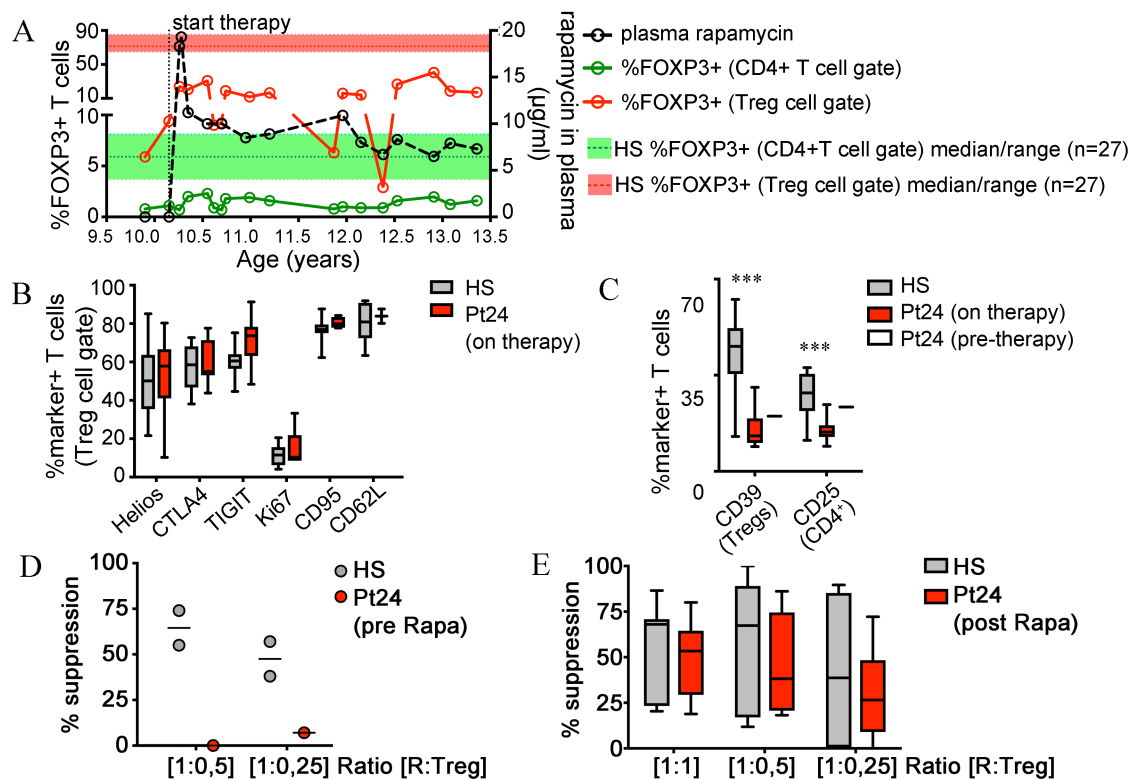
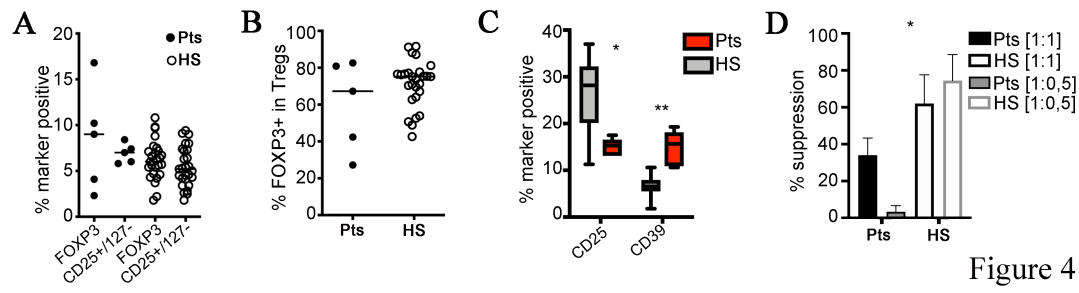


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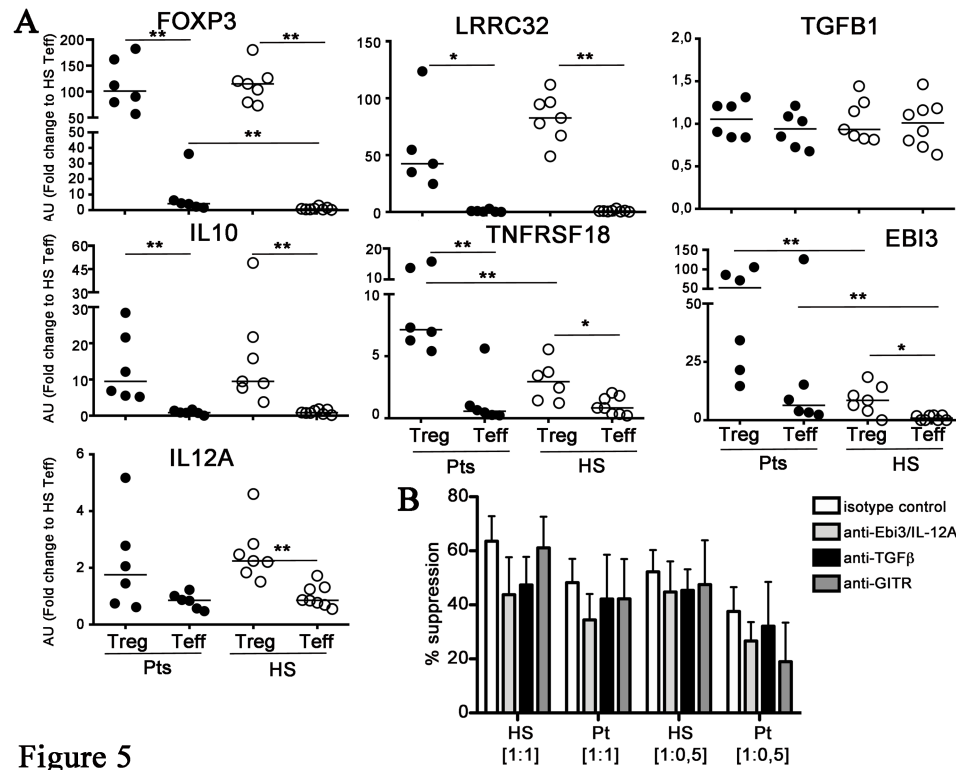


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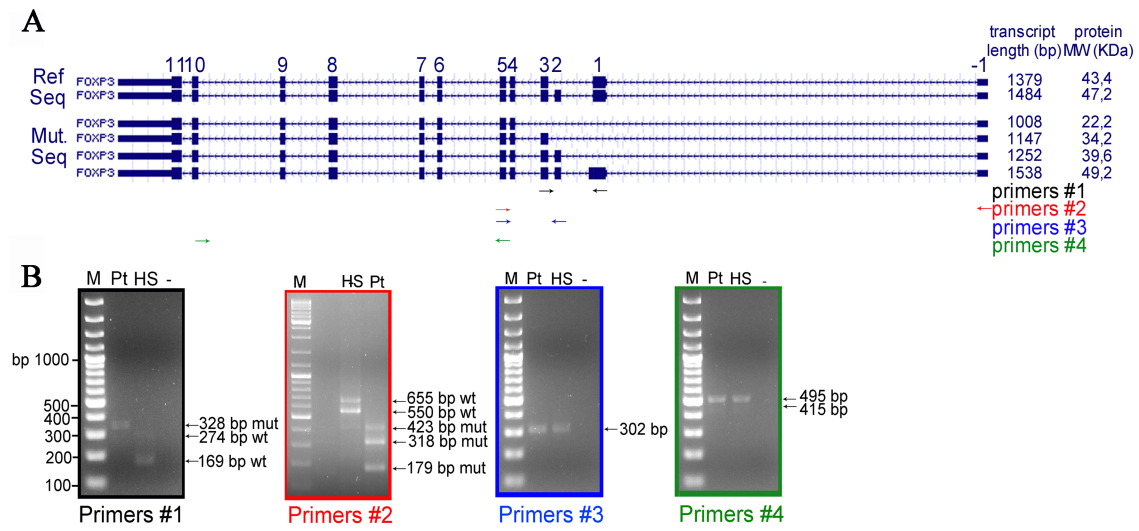


Figure E1

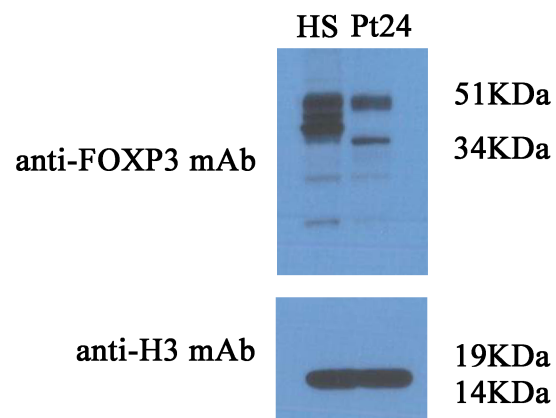


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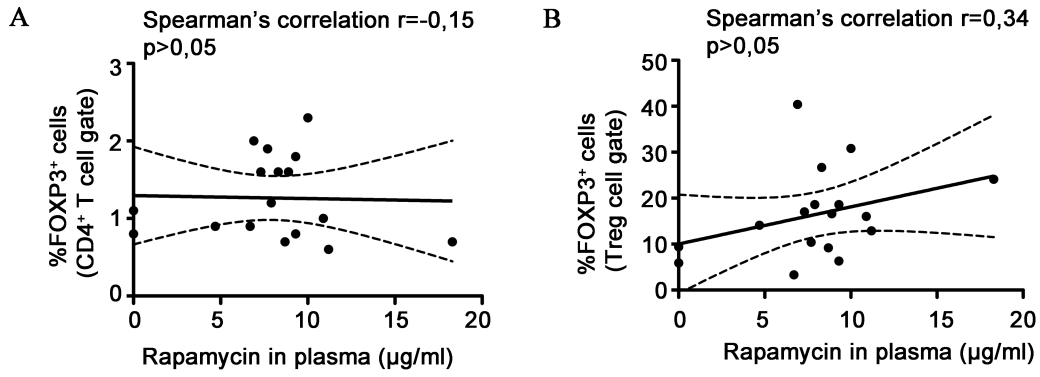


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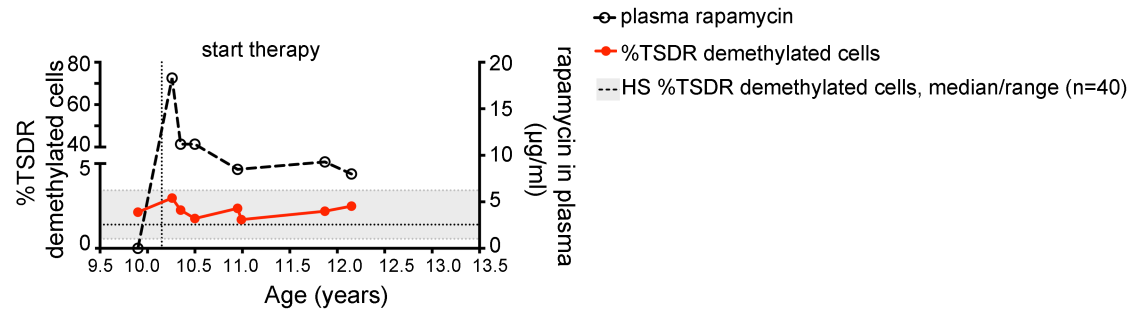


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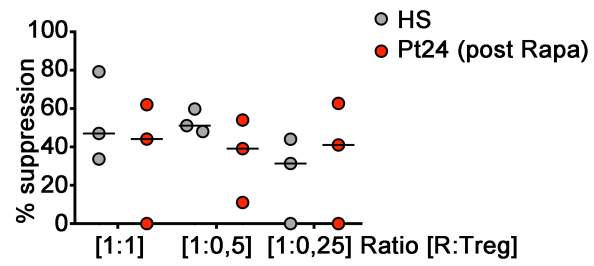


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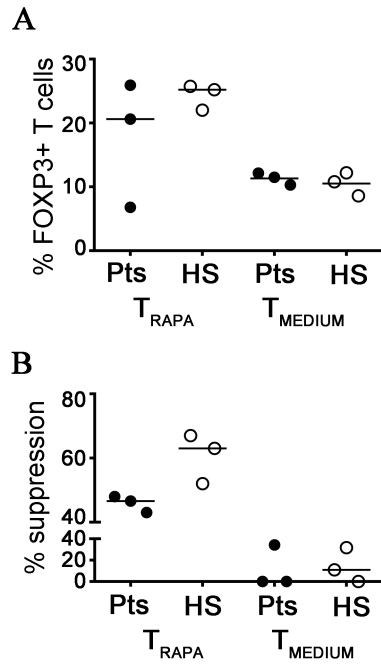


Figure E6

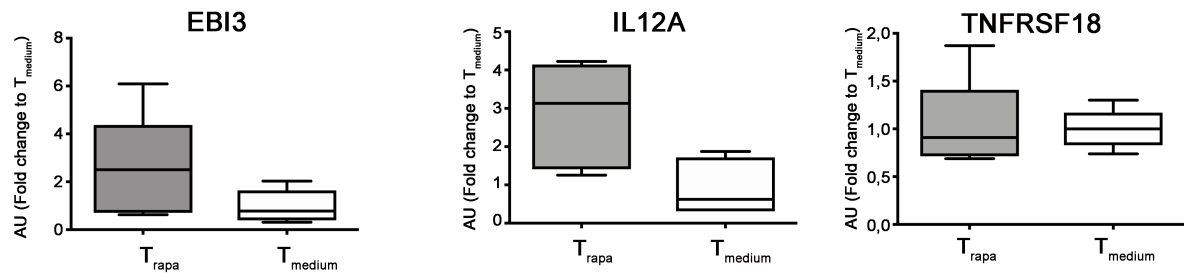


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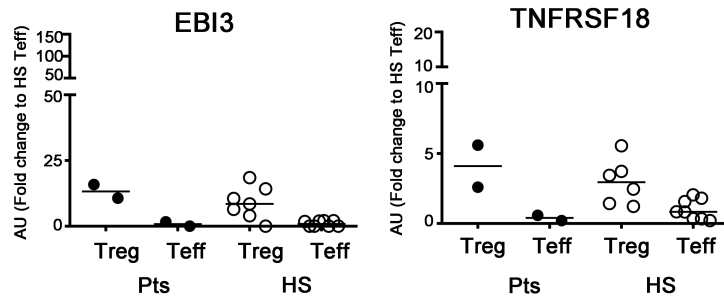


Figure E8

Supplementary Materials

Supplementary Material and Methods:

Western blotting

Polymerase chain reaction

Statistical Analyses

Supplementary Figures:

Fig. E1. FOXP3-mutation c.210+1G>C results in aberrant mRNA splicing isoforms.

Fig. E2. Aberrant FOXP3 isoform expression in IPEX Pt24 peripheral blood.

Fig. E3. FOXP3 expression in peripheral CD4⁺ T cells does not significantly correlate with sirolimus plasma levels.

Figure E4. Normal frequency of circulating TSDR-demethylated tTregs during Rapamycin therapy.

Fig.E5.Rapamycin-conditioned IPEX Tregs display in-vitro suppressive function vs autologous responder cells.

Fig. E6. IPEX CD4⁺ T cells expanded in vitro in the presence of rapamycin acquire suppressive ability.

FigE7.T_{rapa} cells from HS upregulate IL12A and Ebi3 expression.

FigE8. Normal expression of Ebi3 and GITR mRNA in Tregs from IPEX patients devoid of Rapamycin treatment.

Supplementary Tables:

Table E1. Clinical features of patients with IPEX syndrome under Rapamycin therapy.

Table E2. Treg cell phenotype in peripheral blood of IPEX patients under Rapamycin treatment

Table E3. Gene expression analysis – fold change to HS Teff cells.

Table E4. Gene expression analysis – relative expression.

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Supplementary Materials:**Supplementary Materials and Methods***Polymerase chain reaction*

To detect different FOXP3 mRNA isoforms non-quantitative RT-PCR was performed using cDNA from activated PBMC of Pt24. Primer pairs were designed spanning different coding regions (sequences available upon 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) (FigE1). PCR products were run onto 2% Agarose gels (Euroclone) to assess amplicon length and sequenced by Sanger sequencing (Primm) to reconstruct the mutated isoforms.

Western blotting

Cells were lysed in lysis buffer (1%SDS, 10mM HEPES and 2mM EDTA pH7.4), heated at 95°C for 5 min and sonicated. Proteins (10µg per lane) were separated onto NuPAGE® (4–12%) Bis-Tris/MOPS (Thermo-Fisher) and transferred onto nitrocellulose filters (Biorad). After blocking in TBS with 0.05%TWEEN 20 plus 5% non-fat dried milk, membranes were probed with mouse anti-human FOXP3 (Biolegend), followed by goat anti-mouse-IgG-HRP Ab (Dako). Rabbit anti-human anti-H3 mAb (AbCam) was used as control for loading, followed by rabbit anti-mouse-IgG-HRP Ab (Dako).

Statistical Analyses

To evaluate the correlation between the frequency of FOXP3-expressing cells and the plasma levels of rapamycin the non-parametric Spearman's rank correlation coefficient was used. $p < 0,05$ was considered significant.

References

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Online Repository Figure Legends

Figure E1. *FOXP3*-mutation c.210+1G>C results in aberrant mRNA splicing isoforms. **A**, Reference sequences of wt*FOXP3* encoding mRNA (UCSC Genome Browser) and reconstruction of the aberrant splicing isoforms detected in RNA from activated PBMC of Pt24, carrying the genomic mutation 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 PBMC of Pt24 and a HS; relative PCR products are shown. Single amplicons were subjected to Sanger sequencing, reconstruction of the corresponding transcripts detected in Pt24 is reported in B. Mutated isoform #4 is likely to be expressed at extremely low level, since it can be detected only when specific primers are used (primers#1). Numbers on the right indicate the length of the amplicons.

Figure E2. Aberrant *FOXP3* isoform expression in IPEX Pt24 peripheral blood. PBMC from HS or Pt24 were activated for 72 hours in the presence of anti-CD3 and anti-CD28 mAbs, prior to cell lysis and protein extraction. 10µg of total proteins were loaded per lane of a poly-acrylamide gel. After transfer onto nitrocellulose filters, membranes were probed with mouse anti-human *FOXP3* mAb (upper panel) and anti-human H3 mAb (lower panel). Molecular weight was determined based on pre-stained molecular weight marker run in parallel. HS: healthy subject; Pt: patient.

Figure 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 Pt24 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's rank correlation coefficient (n=17 independent measurements). Correlation coefficients are indicated in the graphs.

Figure E4. Constant frequency of circulating TSDR-demethylated tTregs during Rapamycin therapy. The frequency of tTregs in PB was measured by molecular TSDR assay and is reported as % 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 HS¹ (median and range n=40).

Figure E5. Rapamycin-conditioned IPEX Tregs display *in vitro* suppressive function vs autologous responder cells. The suppressive ability of Pt24 Tregs was tested several times during rapamycin therapy at different [R:Treg] ratio using patient's CD4⁺CD25⁻ Teff cells as Responders (R). %suppression median/range was: 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; 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. R: responder cells; Pt: patient; HS: healthy subject.

Figure 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 100nM. After three 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; Pts T_{rapa} 20,6%, <6,8-25,9>; Pts T_{medium} 11,5% <10,3-12,1>, n=3) and **B**, *in vitro* suppression assay using HS PBMC as responder cells at [R:S] ratio of [1:1] were performed (% nhibition of CD4⁺ T cell proliferation median, <range> was: 63,0%, <52,0-67,0>at [R:HS T_{rapa}]=[1:1]; 46,6%, <43,0-48,0>

at [R:Pts T_{rapa}]=[1:1], n=3). Lines in the graphs indicate median values. Pts: patients; HS: healthy subjects; T_{rapa}: CD4⁺ T cells expanded *in vitro* with rapamycin and IL-2; T_{medium}: control CD4⁺ T cells expanded *in vitro* with IL-2 alone; R: responder cells. Clinical and molecular details of Pt20 were previously described in ^{2,3}; Pt26 is an adult IPEX patient (*FOXP3* mutation R347H) devoid of IS therapy at time of sampling.

Figure E7. T_{rapa} cells from HS upregulate IL12A and Ebi3 expression. Ebi3, IL12A (p35) and TNFRSF18 mRNA expression in HS CD4⁺ T cells after three rounds of *in vitro* stimulation in the presence (grey 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> vs T_{medium} 0,8, <0,3-2,0> fold change to average T_{medium} cells; IL12A (p35): T_{rapa} 3,1, <1,3-4,2> vs T_{medium} 0,6, <0,3-1,9>; TNFRSF18 T_{rapa} 0,9, <0,7-1,9> vs T_{medium} 1,0, <0,7-1,3>, n=5, p=ns, Non-parametric Mann-Whitney. AU: Arbitrary Units.

Figure E8. Normal expression of Ebi3 and GITR mRNA in Tregs from IPEX patients devoid of Rapamycin treatment. The expression of EBI3 and TNFRSF18 mRNA was detected by ddPCR using RNA extracted from CD4⁺CD25⁺CD127^{low} Tregs and CD4⁺CD25⁻ Teff FACS-sorted from PB of n=2 IPEX patients who were not under Rapamycin treatment at the time of analysis (Pts 29 – sibling of Pt28, with no clinical manifestations of the disease and devoid of therapy at the time of analysis - and Pt30, TableE1) and HS (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.

Table E1. Clinical features of patients with IPEX syndrome under Rapamycin therapy.

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

Rapa: Rapamycin; Predn.: Prednisolone; Aza: Azathioprine; mo: months; ys: years; FTT: failure to thrive.

*Clinical and immunological data refer to the indicated therapy duration.

°Tapering ongoing.

Table E2. Treg phenotype in PB of IPEX patients under Rapamycin treatment. Frequency of the indicated T cell population was measured by flow cytometry in PB of IPEX patients during Rapamycin therapy and HS. Data are reported as % marker positive cells, median and range, in the indicated cell gate.

ns: not significant; Non-parametric Mann-Whitney.

Table E3. Gene expression analysis – fold change to HS Teff cells. Droplet digital (dd) PCR analysis of gene expression on sorted peripheral $CD4^+CD25^+CD127^{low}$ Tregs $CD4^+CD25^-CD127^+$ Teff cells from IPEX patients with ongoing rapamycin therapy and HS. Data are expressed as AU (fold change to the average expression in HS Teff cells). Median values and range of each target gene are listed.

Table E4. Gene expression analysis – relative expression. Droplet digital (dd) PCR analysis of gene expression on sorted peripheral $CD4^+CD25^+CD127^{low}$ Tregs and $CD4^+CD25^-$ Teff cells from IPEX patients with ongoing rapamycin therapy and HS. Data are expressed as number of copies/ μ l of the test gene normalized to number of copies/ μ l of PGK, used as housekeeping gene for normalization of samples. Median expression/range of each target gene are listed.

Table E1. Clinical features of patients with IPEX syndrome under Rapamycin therapy. Patients were treated with rapamycin alone or in combination with other IS agents.

Pt	Mutations	FOXP3 protein expression	Ongoing Therapy at sampling	Duration of therapy	Major autoimmune manifestations		IgE UI/ml	Lympho /mmc	Treg % of CD4	Eos /mmc	HAA/ VAA
					pre Rapa	on Rapa					
Pt24	c.210+1G>C	no	Rapa	3ys	gastritis, FTT	Solved gastritis	20.0	3600	4.9	800	pos/ neg
Pt25	p.R347H (c.1040G>A)	yes	CsA° Rapa	10mo 7mo	diarrhea, FTT, eczema	Solved diarrhea	0.4	1900	7.4	380	pos/ nt
Pt27	p.V480M (c.1222G>A)	yes	Rapa Predn Anti-TNFα	16mo 4ys 4ys	diarrhea, FTT	Recurrence of chronic diarrhea	n.a.	3180	5.8	90	pos/ neg
Pt28	p.R397Q (c.1190G>A)	yes	Steroids° Rapa Aza	11mo 24mo 8mo	Diarrhea, thyroidis	Solved diarrhea	80.3	5800	7	200	pos/ neg
Pt30	c.736-1G>A	yes	Rapa	2mo	diarrhea, FTT	Improved diarrhea	n.a.	2340	8.4	10	pos/ pos
Pt31	p.R337Q (c.1010G>A)	yes	Steroids Rapa Rituximab	2mo 2mo 4doses	Diarrhea, T1D, eczema, nephropathy	Improved diarrhea	n.a.	2725	6	408	pos/ neg

Rapa: Rapamycin; Predn.: Prednisolone; Aza: Azathioprine; mo: months; ys: years; FTT: failure to thrive; °Tapering ongoing; n.a. not available; n.t.: not tested.

Table E2. Treg phenotype in PB of IPEX patients under Rapamycin treatment.

Markers (gating strategy)	IPEX Pts (n=5) %positive Median (range)	HS (n=16/27) %positive Median (range)	p
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)	0,01
CD39⁺ (CD4⁺)	15,7 (10,6-19,3)	6,6 (1,8-10,6)	<0,0001

Table E3. Gene expression analysis – fold change to HS Teff cells.

Gene target	IPEX Treg (n=6)	HS Tregs (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
IL12A	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	8,5/0-18,5	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
IL10	9,5/5,2-28,4	9,5/3,8-49,0	0,8/0,0-1,7	0,8/0,2-1,8

Table E4. Gene expression analysis – relative expression.

Gene target	IPEX Treg	HS Tregs	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
IL12A	0,015/ 0,006-0,028	0,022/ 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
IL10	0,009/ 0,005-0,027	0,009/ 0,004-2,953	0,001/ 0,000-0,002	0,001/ 0,000-0,002