



IPEX due to an exon 7 skipping *FOXP3* mutation with autoimmune diabetes mellitus cured by selective T_{Reg} cell engraftment

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ABSTRACT

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare inherited disorder leading to severe organ-specific autoimmunity. IPEX is caused by hemizygous mutations in *FOXP3*, which codes for a master transcription factor of regulatory T (T_{Reg}) cell development and function.

We describe a four-year-old boy with typical but slightly delayed-onset of IPEX with autoimmune diabetes mellitus, enteropathy, hepatitis and skin disease. We found the unreported *FOXP3* splice site mutation c.816 + 2T > A that leads to the loss of leucine-zipper coding exon 7. RNA-Seq revealed that *FOXP3*Δ7 leads to differential expression of *FOXP3* regulated genes. After myeloablative conditioning the patient underwent allogeneic HSCT from a matched unrelated donor. HSCT led to the resolution of all IPEX symptoms including insulin requirement despite persisting autoantibody levels. After initial full donor engraftment nearly complete autologous reconstitution was documented, but donor-derived T_{Reg} cells persisted with a lineage-specific chimerism of > 70% and the patient remained in clinical remission.

1. Introduction

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is an inborn error of immunity particularly characterized by severe immune dysregulation [1]. IPEX is caused by hemizygous mutations of the *FOXP3* gene, which codes for the FOXP3 master transcription factor of regulatory T (T_{Reg}) cell development and function [2]. Thus in IPEX, missing or dysfunctional T_{Reg} cells lead to destructive organ-specific autoimmunity [3]. Typical symptoms include autoimmune enteropathy, diabetes mellitus, thyroiditis and skin disease alone or in combination with further autoimmune manifestations [4]. Treatment by pharmacological immune suppression is often insufficient to control autoimmunity and causes side effects and complications. IPEX patients are also prone to infections that are partially attributable to concomitant immunosuppressive treatment [5]. The only curative treatment available at present is allogeneic hematopoietic stem cell transplantation (HSCT) [6,7]. While especially enteropathy and skin disease resolve when donor engraftment is established, damage to other organs can be permanent especially with persistence of diabetes mellitus if post-mitotic pancreatic islet beta cells are lost [4,8]. Systematic

evaluation of HSCT outcome is lacking for larger patient cohorts, but several case reports and small cohorts have reported results after myeloablative and reduced intensity conditioning [4,6,9]. It has been shown that mixed chimerism can cure IPEX and that even selective engraftment of donor regulatory T cells may suffice [10]. However, outcome is still not universally good with a persistent risk of severe complications, especially in older patients [6]. It is therefore mandatory to establish an early diagnosis. However, this can be demanding since there is no clear genotype-phenotype correlation and similar mutations can lead to differential clinical outcomes [5]. Furthermore, dysfunctional FOXP3 proteins can be expressed and phenotypically unsuspicious T_{Reg} cells can be detected by flow cytometry that still are devoid of proper function [5,11]. Therefore, in-depth analysis of individual *FOXP3* variants and their consequences for T_{Reg} cell phenotype and function are necessary to truly establish the diagnosis of IPEX. Over 60 mutations have been described with the majority of them clustering around the FOXP3 forkhead domain necessary for DNA binding [2]. The ongoing discovery of new mutations still increases our knowledge about the physiological importance of other FOXP3 domains [12,13].

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2. Methods

2.1. Subjects

Blood from patient and healthy donors was obtained after written informed consent in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee of the LMU München.

2.2. Isolation and stimulation of peripheral blood T cell populations

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by Biocoll (Biochrom, Berlin, Germany) density gradient centrifugation. Untouched CD4⁺ T cells were separated using the CD4⁺ T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Samples from healthy donors were depleted from T_{reg} cells using the CD4⁺CD25⁺ regulatory T cell Isolation Kit (Miltenyi Biotec). CD4⁺ T cells were stimulated for 48 h with CD3/CD28 beads (Thermo Fisher Scientific, Waltham, USA) at a cell-to-bead ratio of 8:1 in a 24-well microplate in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS (Thermo Fisher Scientific) at a density of 10⁶ cells per well.

2.3. Flow cytometry

Surface staining was performed using anti-CD3-APC-H7 (SK7, AB_1645475), anti-CD4-APC (SK3), anti-CD25-PE (M-A251, AB_395826) (all BD Biosciences, San Jose, USA) and anti-CD127-FITC (Thermo Fisher Scientific, AB_1907342). Intracellular staining of FOXP3 was performed with anti-FOXP3-PerCP-Cy5.5 (PCH101, AB_10854725) after fixation and permeabilization (Thermo Fisher Scientific). Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, USA).

2.4. Genetic analysis of FOXP3

Genomic DNA and total RNA were isolated from CD4⁺ T cells by using QIAzol lysis reagent (Qiagen, Hilden, Germany) and standard ethanol precipitation. Sequencing of all exons and exon-intron boundaries of the FOXP3 gene was carried out using the primers specified in Supplementary Table 1. FOXP3 cDNA was reverse transcribed (QuantiTect, Qiagen) from CD4⁺ T cell total mRNA and amplified by PCR with the following primer pair: forward (5'GCGTCTAGAATGCCAACCAGGCTGGCAAG3') and reverse primer (5'GCGGCGGCCGCTCAGGGGCCAGGTGTAGGTTGGAAC3'). PCR fragments were separated by agarose gel electrophoresis. Skipping of Exon 7 was confirmed by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany).

2.5. Immunoblotting

Whole cell lysates were prepared of unstimulated and anti-CD3/CD28 stimulated CD4⁺CD25⁺ T cells. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-FOXP3 (ebio7979, Thermo Fisher Scientific, AB_468500), anti-γ-Tubulin (GTU-88, Sigma Aldrich, St. Louis, USA, AB_532292) and HRP-conjugated secondary antibody (sc-2005, Santa Cruz, Dallas, USA, AB_631736).

2.6. Illumina RNA-Seq and data analysis

Total RNA was isolated from resting CD4⁺ T cells and after stimulation for 48 h with CD3/CD28 beads using QIAzol (Qiagen) and miRNeasy Mini kit (Qiagen). RNA purity based on A260/A280 ratio and RNA quantity was measured using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). RNA integrity (RIN) was

calculated by using Agilent Bioanalyzer 2100 with an RNA 6000 Pico Kit (Agilent, Santa Clara, USA) and ranged from 8.9 to 10.

Total RNA (100 ng) was treated with double-strand specific DNase (Thermo Fisher Scientific) to remove any traces of genomic DNA. cDNA was synthesized and converted to Illumina-compatible sequencing libraries with the Encore complete RNAseq kit (NuGen, San Carlos, USA). First strand cDNA was generated by random priming with a set of selected hexamers that avoid priming of rRNA, second strand cDNA was synthesized with incorporation of dUTP and the double stranded cDNA was fragmented using a Bioruptor nextgen (Diagenode, Liege, Belgium) sonicator applying 25 cycles of low power (30 s on/off) in a cooled water bath. cDNA was end-repaired and ligated to Illumina-adapters and the second-strand selectively removed by uracil deglycosylase. After 18 cycles of PCR the final libraries were quantified on an Agilent 2100 Bioanalyzer and diluted to 10 nM prior to pooling and sequencing. Pooled libraries were sequenced on a single lane in 100 bp single end mode on a HiSeq1500 instrument (Illumina, San Diego, USA).

Raw sequence reads were demultiplexed and mapped to the human genome (hg19 release) using Tophat 2.0 and hg19 gene annotation obtained from iGenomes (Illumina) [14]. Read counts for each gene were obtained by the python script HTseq count [15]. Read counts were then normalized by r-log transformation with the program DESeq2 [16]. Differentially expressed genes were identified by pairwise comparisons with DESeq2 using FDR threshold of 5%.

2.7. Functional interpretation of gene expression profiles

Pathway enrichment analysis was performed with the database for annotation, visualization and integrated discovery (DAVID v6.8) with the databases of BioCarta and KEGG [17,18]. Enriched pathways (P-value < 0.05) were identified using the Ease Score algorithm, a modified Fisher Exact test. FOXP3-bound genes were retrieved from two published human data sets [19,20].

3. Results

3.1. IPEX-related autoimmunity is cured by selective T_{Reg} cell engraftment

We report a male patient with skin disease resembling atopic dermatitis, recurrent pulmonary infections and systemic CMV infection initially presenting at the age of ten months. At three years of age, episodes of protracted diarrhea with enteric villous atrophy and elevated anti-AIE75 (autoimmune enteropathy-related 75 kDa antigen) autoantibodies lead to the diagnosis of autoimmune enteropathy. Simultaneously, the patient developed an autoimmune overlap-syndrome with hepatitis type 2 and cholangitis with positive anti-LC1 (liver cytosolic antigen 1), anti-PR3-ANCA (proteinase 3 anti-neutrophil cytoplasmic antigen) and AMA (anti-mitochondrial antibodies) autoantibodies, respectively. Additionally, an autoimmune diabetes mellitus manifested with high titers of anti-GAD (glutamic acid decarboxylase) and elevated anti-ICA (islet cell antibodies), anti-IA2 (islet antigen 2) and anti-ZNT8 (zinc transporter 8) autoantibodies (Table 1). Further autoantibodies evaluated by panel diagnostics were negative.

Because of high clinical suspicion for IPEX, we performed flow cytometry and found absent T_{Reg} cells in the peripheral blood of the patient (Fig. 1). Next, we performed Sanger sequencing and detected a hemizygous FOXP3 splice site mutation c.816+2T > A that has not been reported before and changes the mandatory T/U at position +2 of the canonical splice donor site of intron 7 (Fig. 3A).

Based on these findings, immunosuppressive treatment was changed from prednisolone and azathioprine to prednisolone and sirolimus, resulting in clinical remission of hepatitis, enteropathy and eczema. At the age of four years reduced toxicity myeloablative allogeneic HSCT (bone marrow) from a 10/10 HLA-matched unrelated donor (MUD) was performed after conditioning with alemtuzumab, fludarabine, treosulfan and thiopeta. Cyclosporine A and mycophenolate mofetil were

Table 1
Autoantibody levels at the time of diagnosis and after HSCT.

	Before HSCT	1 yr post HSCT	3 yrs post HSCT	Unit	Reference value
AMA	Pos.	Neg.	n.d.	n.a.	Neg.
ANCA	Pos.	n.d.	Neg.	n.a.	Neg.
anti-LC1	Pos.	n.d.	Neg.	n.a.	Neg.
anti-IE75	192	32.46	8.77	Ratio	< 10
anti-islet cell	427	160	10	JDF	< 5
anti-GAD	> 2000	1892	> 2000	IE/ml	< 10
anti-IA-2	106	< 10	< 10	IE/ml	< 10
anti-ZnT8	53	< 15	< 10	IE/ml	< 15

AIE75, autoimmune enteropathy-related 75 kDa antigen; AMA, anti-mitochondrial antibodies; GAD, glutamic acid decarboxylase; IA2, islet antigen 2; ICA, islet cell antibodies; LC1, liver cytosolic antigen 1; PR3-ANCA, proteinase 3 anti-neutrophil cytoplasmic antigen; JDF, Juvenile Diabetes Foundation; n.a., not applicable; n.d., not done; neg., negative; pos., positive.

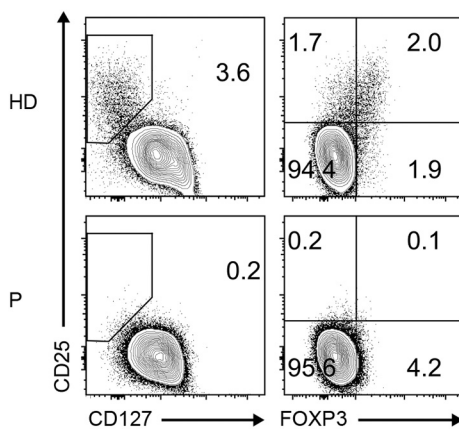


Fig. 1. Absent T_{Reg} cells prior to HSCT. Contour plots of $CD25^{+}CD127^{low}$ and $CD25^{+}FOXP3^{+}$ T_{Reg} cells of a healthy donor (HD) and the IPEX patient (P) after gating on $CD3^{+}CD4^{+}$ T cells. Data are representative for 3 independent experiments.

given as graft-versus-host disease (GVHD) prophylaxis. The post transplantation course was uneventful except for neutropenic bacteremia and asymptomatic CMV reactivation. No acute or chronic GVHD was observed. Importantly, the patient's insulin requirement resolved during conditioning and the autoimmune diabetes remains in remission since then with normal values for blood glucose and HbA1c. Autoimmune enteropathy, hepatitis, cholangitis and eczema remained in remission post HSCT.

Autoantibody levels of anti-LC1, anti-PR3-ANCA, AMA, anti-IA2 and anti-ZnT8 became negative, while anti-AIE75 and anti-ICA continuously decreased after HSCT while anti-GAD remained highly positive (Table 1). Chimerism studies initially revealed a peripheral blood donor chimerism of > 99% up to day +70 with subsequent decrease to < 20%. However, the evaluation of FACS-sorted $CD4^{+}CD25^{+}CD127^{low}FOXP3^{high}$ and MACS-sorted $CD4^{+}CD25^{+}$ T_{Reg} cells revealed an allogeneic fraction of 80–90% and of 40–60% after 2 and 3 years after HSCT respectively (Fig. 2 and Supplementary Fig. 2).

3.2. *FOXP3* splice site mutation c.861 + 2T > A causes skipping of exon 7 (*FOXP3Δ7*)

To further understand the molecular consequences of the novel *FOXP3* splice site mutation c.861 + 2T > A, we analyzed *FOXP3* RT-PCR products of a healthy donor and the patient by gel electrophoresis and Sanger sequencing. As compared to the healthy donor, who expressed the expected *FOXP3* full length (*FOXP3fl*) and physiologic *FOXP3* splice

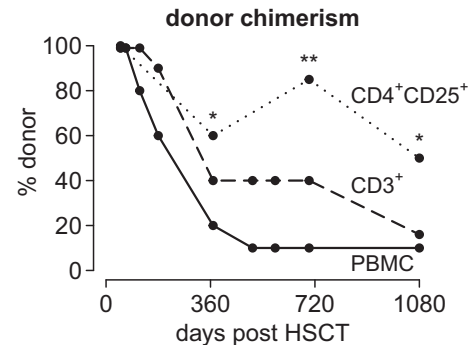


Fig. 2. Chimerism kinetics among PBMC (solid line), $CD3^{+}$ T cells (dashed line) and $CD4^{+}CD25^{+}$ T_{Reg} cells (dotted line) in peripheral blood after HSCT. T_{Reg} cells were sorted by MACS for $CD4^{+}CD25^{+}$ (*) or by FACS for $CD4^{+}CD25^{+}CD127^{low}FOXP3^{high}$ (**).

variant lacking exon 2 (*FOXP3Δ2*) transcripts, we found two shortened *FOXP3* transcripts in the patient, namely *FOXP3* lacking exon 7 (*FOXP3Δ7*) and *FOXP3* lacking exons 2 and 7 (*FOXP3Δ2Δ7*) (Fig. 3B and Supplementary Fig. 1). For comparison, the genomic architecture of the *FOXP3* locus and the exon structures of the detected *FOXP3* variants of the healthy donor and the patient are illustrated (Fig. 3C).

3.3. *FOXP3Δ7* and *FOXP3Δ2Δ7* proteins are expressed in activated T cells

FOXP3 protein is not exclusively expressed in T_{Reg} cells but also transiently in activated T cells [21]. To analyze the induction and expression of *FOXP3* and of its transcriptional target *CD25*, we stimulated purified healthy donor and patient $CD4^{+}$ T cells with anti-CD3/CD28 and performed flow cytometry. Both intracellular *FOXP3* and membrane-bound *CD25* proteins were absent in resting $CD4^{+}$ T cells of the patient but were expressed after anti-CD3/CD28 stimulation in comparable cell fractions and mean fluorescent intensities in healthy donor and patient $CD4^{+}$ T cells (Fig. 4A). To analyze the expression of particular *FOXP3* protein isoforms, we used the same stimulated cells for total protein extraction and *FOXP3* immunoblotting. In line with the *FOXP3* transcripts detected before (Fig. 3B), we identified *FOXP3* protein doublets with different sizes in healthy control and the patient $CD4^{+}$ T cells (Fig. 4B) that most likely correspond to *FOXP3fl* and *FOXP3Δ2* or *FOXP3Δ7* and *FOXP3Δ2Δ7* protein isoforms, respectively. Interestingly, the overall *FOXP3* expression in the patient was elevated as compared to the control (Fig. 4B and C) [22].

3.4. *FOXP3Δ7* alters gene expression in resting and stimulated T cells

FOXP3 orchestrates activating and repressive transcriptional programs by forming homo- and heterodimers with numerous transcriptional cofactors [23,24]. IPEX-causing *FOXP3* mutations affect different protein domains and provide information about their particular functions. *FOXP3* exon 7 is part of the leucine zipper domain and is required for homodimerization and interaction with FOXP1 and Histone H1.5 [25,26]. These heterodimers cooperatively transactivate target genes such as *IL2* and *CTLA4* [23,24]. To identify differential gene expression patterns between *FOXP3fl* and *FOXP3Δ7* $CD4^{+}$ T cells, we stimulated T_{Reg} cell-depleted $CD4^{+}$ T cells from healthy donors and the T_{Reg} cell-deficient $CD4^{+}$ T cells from the IPEX patient with anti-CD3/CD28 and performed RNA-Sequencing (RNA-Seq) on a HiSeq1500 Illumina platform. Differential regulated genes in resting ($n = 3281$) and in stimulated ($n = 1.908$) $CD4^{+}$ T cells were assigned to particular signaling pathways using DAVID bioinformatics resources (Fig. 5A, gene sets with an adjusted p-value < 0.05 are listed in Supplementary Table 2). Notably, 25 signaling pathways related to immune function such as T cell receptor-, cytokine-, FoxO-, CD40L-, NFκB-signaling and T_H1/T_H2 -

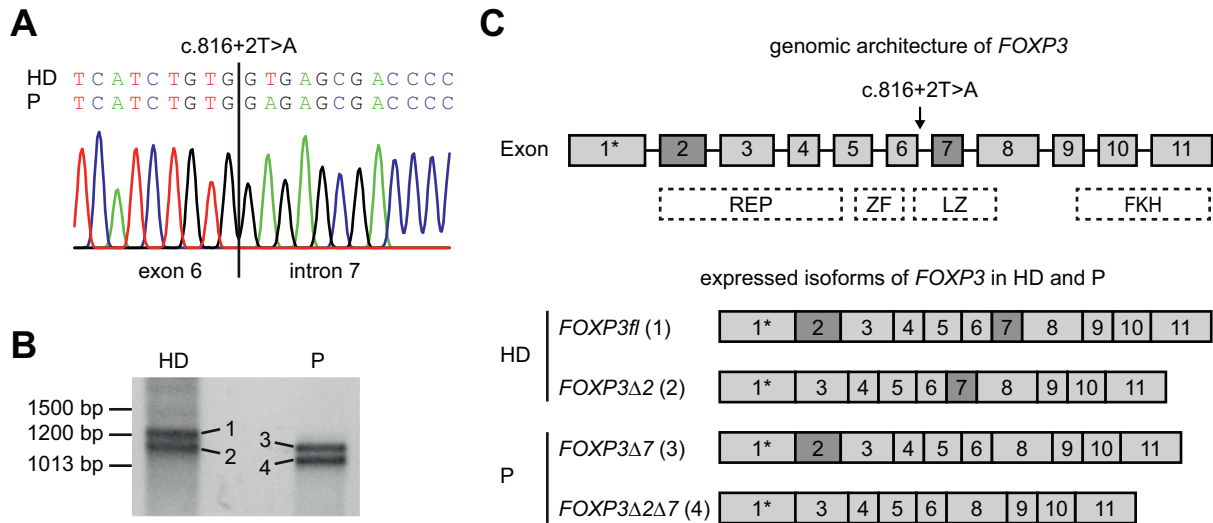


Fig. 3. The *FOXP3* c.816 + 2T > A splice site mutation leads to skipping of exon 7. (A) Sequence alignment and/or electropherogram of healthy donor (HD) and IPEX patient (P) *FOXP3* exon 6 and intron 7 boundary indicating the c.816 + 2T > A splice site mutation. (B) Agarose gel electrophoresis of RT-PCR products of *FOXP3* from a healthy donor (1 and 2) and the IPEX patient (3 and 4). PCR products 1 and 2 correspond to *FOXP3*fl (full-length *FOXP3*) and *FOXP3*Δ2 isoform. PCR products 3 and 4 correspond to *FOXP3*Δ7 and *FOXP3*Δ2Δ7. Detailed sequencing results are shown in Supplementary Fig. 1. (C) Schematic representation of the genomic *FOXP3* organization and corresponding protein domain architecture with the localization of the c.816 + 2T > A splice site mutation found in the IPEX patient. An asterisk is indicating the first coding exon of *FOXP3*.

differentiation were enriched in the patient CD4⁺ T cells (Fig. 5B, the complete pathway lists are available in Supplementary Table 2).

To specifically identify *FOXP3* target genes that react to the loss of the *FOXP3* leucine zipper domain, we analyzed the proportion of differentially regulated genes that have already been identified as *FOXP3* target genes (n = 7845) by Birzele et al. and Sadlon et al. [19,20]. We identified 1938 differentially regulated *FOXP3* target genes in resting, 2433 in stimulated patient and 1948 in stimulated healthy donor CD4⁺ T cells that are related to the loss of the *FOXP3* leucine zipper domain. Within the group of identically regulated genes (n = 3935) in stimulated patient and healthy donor CD4⁺ T cells, 1322 *FOXP3* target genes were identified that are not affected by the loss of the leucine zipper domain (Fig. 5B and Supplementary Table 2). These findings essentially reflect transcriptional and signaling pathway deregulation in *FOXP3*Δ7 CD4⁺ T cells that are particularly associated with abnormal expression of *FOXP3* regulated genes i.e. of *IL2RA*, *CTLA4*, *NFKB1*, *FOXO3*, *RORC*, *HIF1A*, *IL2*, *IL17* and *IL22*.

4. Discussion

It is well accepted that *FOXP3* is essential for the development and

function of T_{Reg} cells and that its absence leads to IPEX syndrome [27]. Four naturally occurring *FOXP3* splice variants, i.e. *FOXP3*fl, *FOXP3*Δ2, *FOXP3*Δ7 and *FOXP3*Δ2Δ7, have been described in humans [28,29]. However, little is known about their individual involvement in human T_{Reg} cell development and function. We here report the *FOXP3* splice site mutation c.861 + 2T > A that only leads to expression of *FOXP3* transcripts and *FOXP3* protein isoforms lacking exon 7. The naturally occurring splice variants *FOXP3*Δ2 and *FOXP3*Δ2Δ7 code for functional inhibitors of human CD4⁺ T cell activation and in mice the exclusive expression of *FOXP3*Δ2Δ7 leads to severe autoimmunity [28–30]. We have shown that this is associated with preferential nuclear localization of *FOXP3*Δ2 and *FOXP3*Δ2Δ7 protein isoforms due to loss of nuclear export sequences (NES) in exons 2 and 7 [31]. To date, only few IPEX causing *FOXP3* in-frame deletions within exon 7 have been reported [2]. However, several *FOXP3* intronic mutations including c.816del+2 have been published and we here reported the novel *FOXP3* mutation c.816 + 2T > A that leads to skipping of exon 7 [2,4]. Thus, these findings substantiate the importance of this splice donor consensus site for *FOXP3* transcriptional regulation and the expression of *FOXP3*fl for proper T_{Reg} cell development and function. Additionally, *FOXP3*Δ2 and *FOXP3*Δ2Δ7 might participate in negative regulation of CD4⁺ T cell

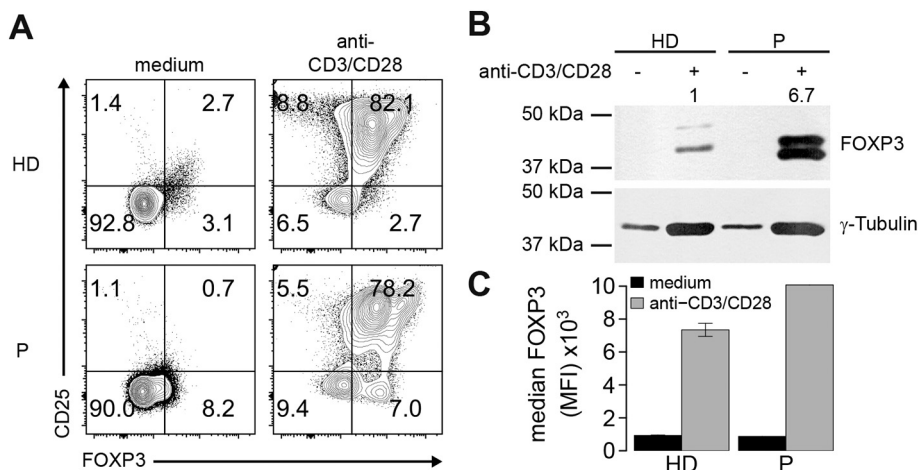


Fig. 4. *FOXP3* protein expression in activated CD4⁺ T cells. (A) Contour plots of CD25 and *FOXP3* expression of healthy donor (HD) and IPEX patient (P) CD4⁺ T cells without (medium) and after stimulation for 48 h with anti-CD3/CD28 and (B) immunoblot of *FOXP3* and the re-probed loading control γ-Tubulin. Relative *FOXP3* expression of P CD4⁺ T cells was determined by densitometry of total *FOXP3* normalized to γ-Tubulin and to expression levels in HD CD4⁺ T cells. Data are representative of two independent experiments. (C) Summary of median *FOXP3* MFI of three different HDs and the IPEX P CD4⁺ T cells analyzed by flow cytometry without (medium) and after stimulation for 48 h with anti-CD3/CD28 (n = 3).

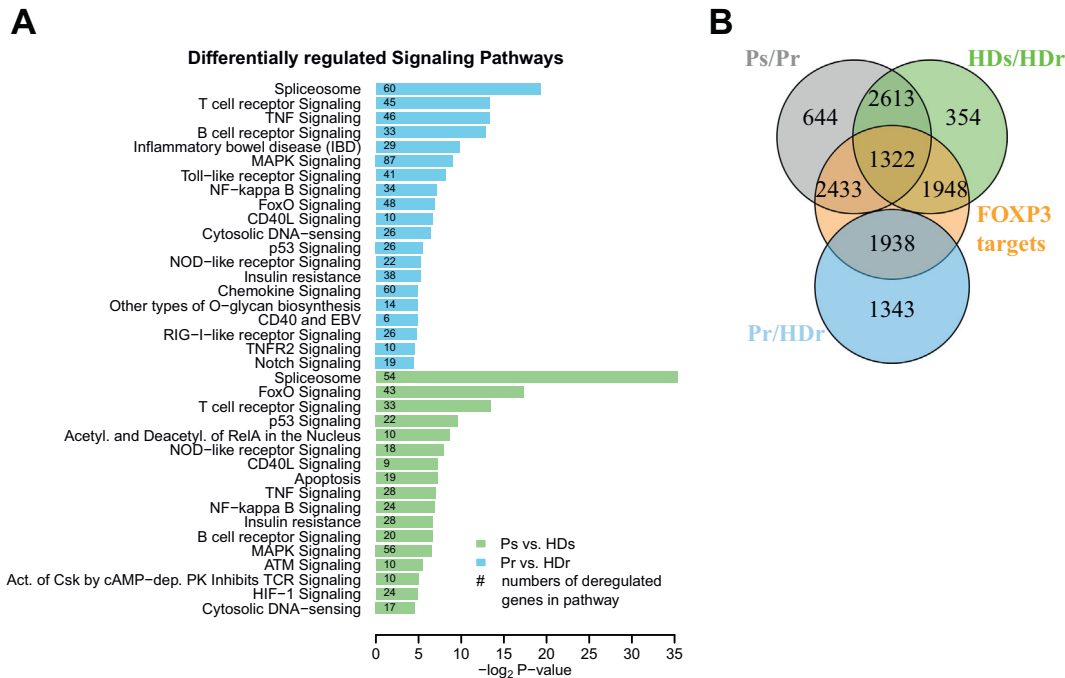


Fig. 5. FOXP3 Δ 7 alters gene expression in resting and stimulated CD4⁺ T cells. (A) Pathway analysis of differentially expressed genes using DAVID bioinformatics resources with BioCarta and KEGG databases [17,18]. Enriched pathways (Fisher's exact P-value < 0.05) related to immune function are shown. The complete pathway lists are available in Supplementary Table 2. Blue bars indicate enriched pathways of differentially regulated genes in resting and green bars in anti-CD3/CD28 stimulated CD4⁺ T cells. Numbers in each bar represent frequency of differentially regulated genes. (B) Integration of differentially expressed genes in resting (Pr/HDr and blue), stimulated IPEX patient (Ps/Pr and grey) and stimulated (HDr/HDs and green) healthy donor CD4⁺ T cells with FOXP3 target genes published by Birzele et al. and Sadlon et al. (orange) [19,20]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and T_{Reg} cell development and function. As the IPEX patient presented here was completely devoid of CD4⁺CD25⁺CD127^{low}FOXP3⁺ T_{Reg} cells we conclude that FOXP3 exon 7 is involved in T_{Reg} cell development.

The FOXP3 leucine zipper domain is partially encoded by exon 7 and is required for homo- and heterodimerization with FOXP1 and histone H1.5 to cooperatively modulate the expression of T_{Reg} cell target genes such as *IL2* and *CTLA4* [23–26]. The most important dimerization interactions are centered around amino acids 250–252 and targeted mutagenesis interferes with the transcriptional repressor function of FOXP3 [32,33]. The FOXP3 exon 7 downstream amino acid sequence mediates interaction with AML1/RUNX1 and this heterotrimer down-regulates pro-inflammatory cytokines and propagates T_{Reg} cell suppressive functions [34]. Although we are not able to completely predict the effects of loss of FOXP3 exon 7 on T_{Reg} cell biology, the relatively mild phenotype of IPEX patients with exon 7 splice site mutations might suggest a residual T_{Reg} cell function [35,36]. This is mirrored by the presented RNA-Seq data, which show several unaffected FOXP3 target gene expression levels and suggest some FOXP3 transcriptional activity independent of exon 7. Still, the IPEX phenotype per se is typical, and again this is reflected by the altered transcriptional status of numerous FOXP3 targets genes including key regulators of CD4⁺ effector T and T_{Reg} cell development and function that are distributed over multiple signaling pathways such as T cell receptor-, cytokine-, FoxO-, CD40L-, HIF-1-, NFκB-signaling and T_H1/T_H2/T_H17-differentiation. Although cellular defects in IPEX are mainly attributed to T_{Reg} dysfunction there is evidence that changes in CD4⁺ effector T cell function also contribute to the phenotype [21,37]. Notably, we identified numerous deregulated genes involved in T_H17 cell differentiation such as IL-6, IL-17, IL-22, RORC and HIF-1.

FOXP3 expression is not restricted to the T_{Reg} cell compartment given that TCR stimulation induces upregulation of FOXP3 in CD4⁺CD25[−] T cells [21]. In non-T_{Reg} cells, however, induced FOXP3

expression is transient and regulated by polyubiquitination-dependent degradation. Five distinct FOXP3 lysine residues are targeted for ubiquitination with four of them being encoded by exon 7 [22]. We found elevated levels of induced FOXP3 protein levels in the IPEX patient CD4⁺ T cells compared to control cells that might be a direct consequence of reduced ubiquitination acceptor sites in the FOXP3 Δ 7 isoforms impairing ubiquitination-directed proteosomal protein degradation.

Allogeneic HSCT cures IPEX patients and leads to resolution of organ-specific autoimmunity with the frequent exception of autoimmune diabetes mellitus [4]. Mechanistically, autoimmune lymphocytic infiltration and destruction of post-mitotic pancreatic beta cells result in life-long insulin-dependency [8,38]. However, before end-stage beta cell depletion is reached, there seems to be a prior dysfunctional stage that can be stopped by T cell depletion during conditioning and reversed by donor T_{Reg} cell engraftment. For example, the IPEX patient reported by Baud et al. became insulin independent with undetectable anti-GAD and anti-ICA autoantibodies six months after HSCT [7]. Additionally, Rao et al published another IPEX patient with a 50% insulin dose reduction with undetectable anti-GAD autoantibodies after HSCT [39]. Notably, the IPEX patient reported here became insulin independent but preserved anti-GAD and anti-ICA autoantibodies for 3 years after HSCT at levels that generally are predictive for autoimmune diabetes mellitus. This finding underlines that in IPEX autoantibodies are not the driving force of beta cell depletion and emphasizes the major role of T_{Reg} cell dysfunction in the development of IPEX-associated and possibly other forms of autoimmune diabetes mellitus [40].

In IPEX patients donor-derived T_{Reg} cells probably have a survival advantage and after HSCT can preserve remission despite rejection of other hematopoietic cell lineages [10,41,42]. Our patient is the fourth reported case that has remained symptom-free due to selective T_{Reg} cell engraftment. The reported percentages of curative T_{Reg} cell donor

chimerism range from approximately > 80% to > 90% and we now add a case with > 70% [10,42]. However, the minimal T_{Reg} cell donor chimerism necessary for long-term cure of IPEX cannot be easily determined especially because it is possible that the donor T_{Reg} cell repertoire might be more important than T_{Reg} cell numbers or percentages. Still, these results suggest that adoptive transfer of autologous gene corrected T_{Reg} cells might suffice to cure human IPEX [43]. Nevertheless with the delicate balance held by autologous FOXP3-deficient CD4⁺ effector T and selective allogeneic FOXP3-proficient T_{Reg} cells long-term follow up is warranted with a particular focus on T_{Reg} cell chimerism, autoimmune diabetes mellitus and additional IPEX manifestations.

In summary, we report an IPEX patient with a novel *FOXP3* splice site mutation c.816+2T>A that leads to skipping of exon 7 (*FOXP3Δ7*), impairs T_{Reg} cell biology by altering the T cell transcriptome and results in a typical but slightly delayed-onset IPEX phenotype. We further show that remission of autoimmune diabetes is possible after timely HSCT even with selective donor T_{Reg} cell chimerism and despite persistence of specific autoantibodies.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

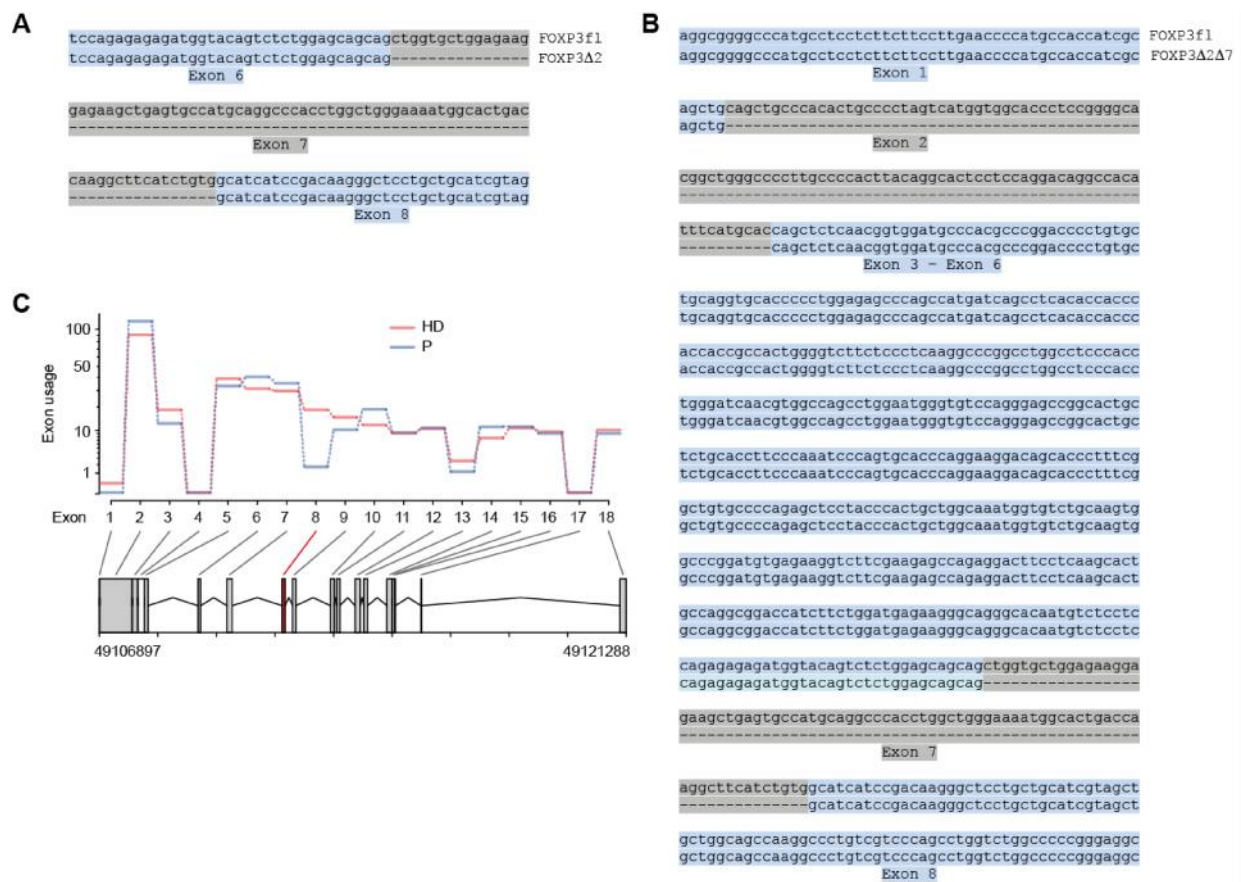
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clim.2018.03.008>.

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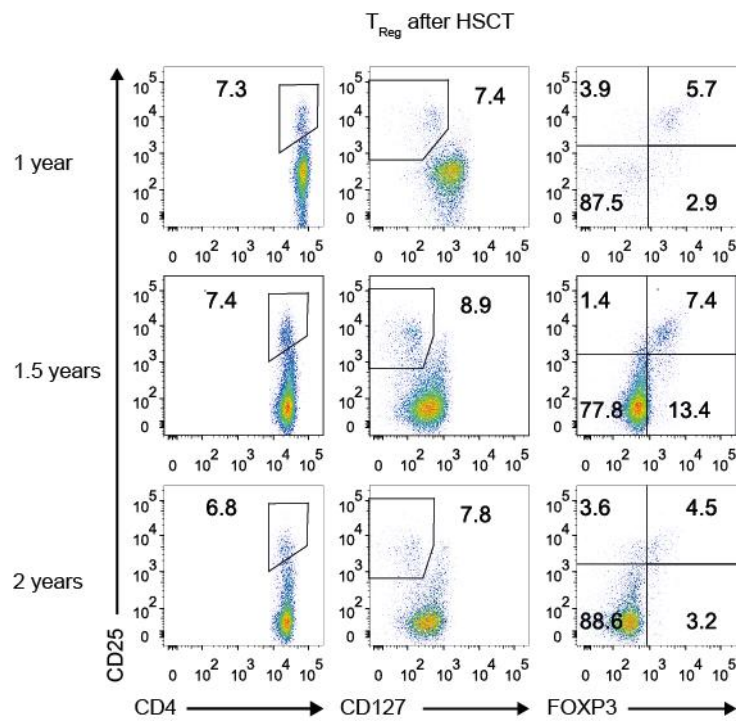
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Supplementary Information



Supplementary Figure 1. *FOXP3* c.861+2T>A leads to skipping of exon 7. Sanger sequencing of *FOXP3* RT-PCR products 2 and 4 (compare Fig. 2B) expressed in the IPEX patient reveal loss of exon 7 expression in *FOXP3f1* (A) and *FOXP3Δ2* (B). (C) The statistical *DEXSeq* method was used to test for differential exon usage in RNA-Seq data. Differential exon usage for *FOXP3* between HD and P is shown in red. Exon 8 refers to exon 7 (note: sequence starts with untranslated exon 1 of *FOXP3*).



Supplementary Figure 2. HSCT restores T_{Reg} population in the patient. Flow cytometry plots show $CD4^+CD25^+CD127^{low}FOXP3^+T_{Reg}$ cells in the patient at various time points after HSCT.

Supplementary Table 1. PCR primers for *FOXP3* amplification.

Exon	Forward primer (5' - 3')	Reverse primer (5' - 3')
2	AGCTGGGGTGCAACTATGGG	CAGGTGGGGATTTTCTTGGC
3-4	TGTGGGCTTGCAGTGCAGAG	CTGAGACCTGACACCTTTGACCC
5-6	AGGCTCAGGAGGGTGGGAAG	GATGGAGCTGGGATTTGAACC
7-8	CCCTCCCCATTGAGAGCATTG	CCTGAATGTGAGGTTAGGTTCCC
9	TGAGCCGAGATTGCACCACTG	TTTTGTGAGCGGATGCATTTTC
10	ACGGGTGTTGACGGTGAGATC	GGGCTTGGGAATGGAGGAAC
11-12	GTCCTGTCCCTGATTACCTGCC	CCTATCATCCCTGCCCCCAC