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FOXP3 expression following bone marrow transplantation for IPEX syndrome after reduced-intensity conditioning

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Abstract The objective of this study is to determine if immune reconstitution of FOXP3+ T regulatory cells correlates with clinical improvement of IPEX syndrome following allogeneic hematopoietic stem cell transplant. An 8-months-old male infant with a mutation in the polyadenylation site of FOXP3 gene, absence of FOXP3 protein expression and clinical manifestations of IPEX syndrome, including eczema, colitis, failure to thrive, TPN requirement, and elevated serum IgE, underwent matched unrelated hematopoietic stem cell transplant. After reduced-intensity conditioning with alemtuzumab followed by fludarabine and melphalan the patient's neutrophils engrafted day +15 and platelets day +29. Patient was a full donor chimera day +28 and +60. Intracellular FOXP3 protein expression in CD4+ T cells was absent pre-HSCT. After transplantation, percentage CD4+ T cells expressing FOXP3+CD25 bright phenotype quickly increased from 4.5 (day +29) to 23% (day +90) and continued in this trend. Foxp3 mRNA expression confirmed flow cytometry data. Serum IgE levels decreased from 5,000 IU/ml pre-transplant to 6 IU/ml on day +90, eczema resolved, and secretory diarrhea and feeding intolerance improved. T regulatory cell reconstitution is evident soon after HSCT following reduced-intensity conditioning correlating with development of full donor chimerism. Increased FOXP3 expression correlates with correction of clinical and laboratory manifestations of IPEX syndrome providing direct evidence that HSCT is a curative procedure for this disorder.

Keywords Immunodeficiency · Transplantation · Regulatory T cells

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Background

The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome is a recessive disorder of early childhood. IPEX is a lethal disorder of autoimmunity caused by mutations in the forkhead box P3 (FOXP3) gene [1]. The most common clinical features of IPEX are severe watery diarrhea and failure to thrive, early-onset insulin-dependent diabetes mellitus, and eczema [1, 2].

The results of immunologic investigations of IPEX are inconsistent with any of the known X-linked immunodeficiency diseases and laboratory findings are remarkably unimpressive. Severe infections have been observed in patients with IPEX [3], but they may have complications of immunosuppressive therapy. There is evidence of immune dysregulation based on marked elevations in IgE and severe food allergy [3]. Furthermore, massive infiltration of T lymphocytes into the skin and gastrointestinal tract and high serum levels of autoantibodies against blood, thyroid, and pancreatic cells suggest that IPEX is an autoimmune disorder [3]. Several mutations of the FOXP3 gene have been identified in patients with IPEX, confirming that IPEX is a distinct X-linked immune disorder. Scurfy mice, in which a disease similar to IPEX develops, have a mutation in an analogous gene [4, 5].

Supportive therapy with total parenteral nutrition, insulin, blood transfusions, and immunosuppressive therapy is beneficial in patients with IPEX. Nevertheless, the prognosis is poor, and most reported cases have been fatal. Successful bone marrow transplantation for IPEX syndrome has been described [2, 6]. Engraftment and amelioration of symptoms in four patients following HSCT using reduced-intensity conditioning has been described by Rao et al. [2].

Case report

A 4½-months-old male was transferred to our hospital under the care of the gastroenterology service for the evaluation of failure to thrive and profuse diarrhea. On two prior occasions, he presented to an emergency department requiring IV fluids for dehydration, and electrolyte imbalance associated with vomiting and diarrhea. The subject was born full term through spontaneous vaginal delivery. Shortly after birth the subject developed severe diarrhea, dermatitis, and failure to thrive. Colonic biopsy revealed severe enteritis. Mutational analysis identified point mutation AATAAA → AATAAG within the polyadenylation site of the FOXP3 gene leading to low mRNA levels and absence of CD4+CD25+ T regulatory cells (Tregs). Family history included a half-sibling who died during infancy due to mutation confirmed IPEX syndrome.

The immunology service was consulted to manage immunosuppression. Initiation of rapamycin, methotrexate, and prednisone offered moderate improvement. He gained weight, but still required TPN due to enteropathy. Despite being on rapamycin, he developed hyperglycemia requiring insulin. Our patient's pre-transplant course was complicated by the development of *Enterobacter cloacae* sepsis necessitating central venous catheter removal.

The patient had white blood cell count 7,610/μl (normal range 6,000–14,000/μl), hemoglobin of 8.8 g/dl (normal range 10.5–14.0 g/dl), and normal platelets. The absolute eosinophil count was elevated at 850/μl. Quantitative assay for immunoglobulins revealed a level of IgG 656 mg/dl (normal range 286–1,058 mg/dl); IgA 49 mg/dl (normal range

10–131 mg/dl); IgM 73 mg/dl (normal range 21–192 mg/dl); and IgE > 5,000 IU/ml (normal range 0–30 IU/ml).

Lymphocyte analysis revealed absolute CD3+ cells 4,355/μl (normal range 2,300–6,900/μl), absolute CD4+ cells 3,010/μl (normal range 1,400–5,100/μl), absolute CD8+ cells 1,255/μl (normal range 600–2,200/μl), absolute CD19+ cells 1,873/μl (normal range 700–2,500/μl), and absolute CD16+/CD56+ cells 426/μl (normal range 100–1,000/μl). Natural killer cell function demonstrated normal lytic activity. Lymphocytes responded greater than tenfold to phytohemagglutinin, concanavalin A, and pokeweed mitogen. His karyotype was normal 46, XY.

Results

Procedure: At the age of 7 months the patient underwent reduced-intensity conditioning with alemtuzumab 30 mg total day −21 to −19, followed by fludarabine 1 mg/kg/day on days −8 to −4 and melphalan 4.7 mg/kg on day −3. GVHD prophylaxis included cyclosporine starting on day −2, methotrexate 10 mg/m^2/day on day +1 and 7.5 mg/m^2/day on days +3 and +6, and prednisone 1 mg/kg/day on day +7 and +28 then slowly tapered. Patient received unmodified bone marrow graft from a 10/10 allele matched unrelated donor with a cell dose of 4.72×10^6 CD34+ cells/kg of recipient weight.

Neutrophil (ANC > 500 cells/μl) and platelet engraftment (platelet count > 50,000 cells/μl) occurred on day +15 and day +27, respectively. He was a full donor chimera on day +28.

Lymphocyte analysis revealed absolute CD3+ cells 2,115/μl (normal range 1,600–6,700/μl), absolute CD4+ cells 1,280/μl (normal range 1,000–4,600/μl), absolute CD8+ cells 763/μl (normal range 400–2,100/μl), absolute CD19+ cells 1,144/μl (normal range 600–2,700/μl), and absolute CD16+/CD56+ cells were 173/μl (normal range 200–1,200/μl). His post transplant course was complicated by the development of biopsy-proven skin graft versus host disease necessitating systemic steroid therapy. He also developed *Staphylococcus epidermidis* bacteremia, which was treated with antibiotics. He developed significant hypertension requiring multiple medications to control.

Immune reconstitution was characterized by using flow cytometry analysis of peripheral blood mononuclear cells using fluorescent antibodies against CD3, CD4, CD8, CD16/56, and CD19. Tregs were quantified by using flow cytometry for CD4+CD25+FOXP3+ cells and semi-quantitative PCR for Foxp3 expression, which was normalized against GAPDH. Day +29 analysis of lymphocytes revealed clear reconstitution of Tregs with 4.94% of CD3+CD4+ T cells bearing CD25+FOXP3+ markers (Fig. 1). Day +90 and day +125 showed marked expansion of Tregs (Fig. 2). Foxp3 RT-PCR was conducted on all time points and revealed detectable expression by day +90 and day +125 (Fig. 2). During this time clinical signs of skin and gut autoimmunity and allergy had resolved (Table 1). Regression analysis of CD4+CD25+CD127− T cells with CD4+CD25+FOXP3+ T cells revealed goodness of fit with $R^2 = 0.8859$, suggesting that CD127 can be a reliable marker of Tregs following HSCT (Fig. 3).

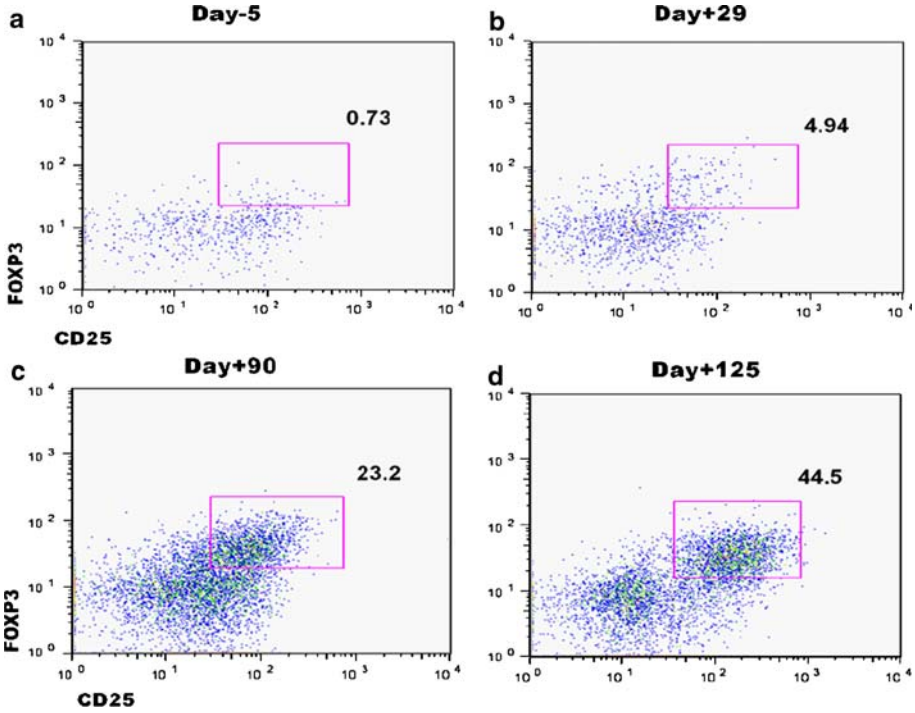

Fig. 1 T regulatory cell reconstitution following HSCT. Subject PBMC's were surface stained for CD3, CD4, and CD25, and intracellular FOXP3. Flow cytometry analysis was conducted on samples collected on **a** day −5, **b** day +29, **c** day +90, and **d** day +125

Discussion

Similar to other reports of patients with IPEX who underwent HSCT following reduced-intensity conditioning, our patient tolerated the procedure well [2, 7]. We monitored Treg reconstitution following HSCT and observed clear evidence of FOXP3 expression in the CD4+CD25+ population increasing over time. Prior to HSCT, no FOXP3 expression could be established in this patient's samples. Our patient showed remarkable resolution of IPEX syndrome associated clinical manifestations, which correlated with increased FOXP3 expression confirmed by both flow cytometry and PCR analysis. CD4+CD25+FOXP3+ Treg cell populations are readily identified by day +29 following bone marrow transplantation for IPEX syndrome after reduced-intensity conditioning. High correlation exists between CD4+CD25+FOXP3+ Treg populations and those bearing CD4+CD25+ CD127− markers and can be used as method of monitoring Treg immune reconstitution. Increased FOXP3 expression correlates with improvement of clinical manifestations of IPEX syndrome in this patient and provides direct evidence that HSCT is a curative procedure for this disorder.

It is of interest to note that Treg numbers expanded significantly in the first several months following transplant. Zhan et al. [7, 9] have noted Treg reconstitution in their IPEX patient after HSCT that followed a fairly consistent ratio of CD4+ T cells and is similar to T cell reconstitution patterns seen in other pediatric allogeneic stem cell transplants. Unlike the subject described by Zhan et al. [7], our patient had no FOXP3 expression, and thus can

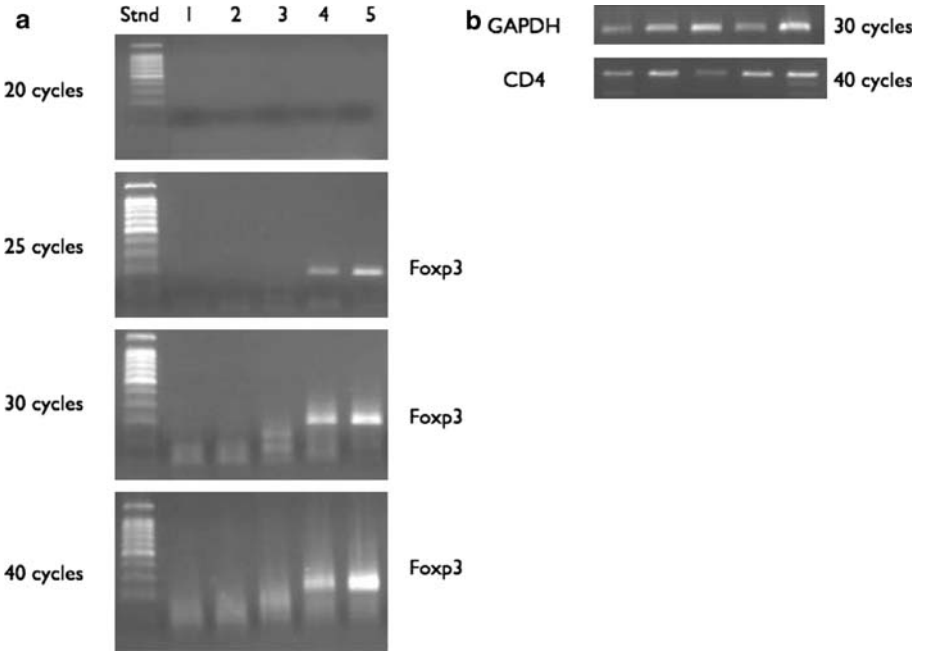

Fig. 2 **a** Foxp3 RT-PCR of patient samples. Leukocytes were harvested from total blood and total RNA was isolated, DNase-treated, and prepared as single strand cDNA. Equal amounts of starting total RNA (150 ng), and subsequently cDNA as template, were used in two rounds of nested PCR to detect Foxp3 expression. After 25 cycles of PCR in the second nested round, Foxp3 expression is strongly detectable in the 4th and 5th collection samples. Even after 40 cycles of nested PCR, Foxp3 expression is not detectable in earlier samples. (The PCR product was sequenced and confirmed as Human Foxp3.) *Column 1* is patient sample day −5, *column 2* is day +29, *column 3* is day +61, *column 4* is day +90, and *column 5* is day +120. **b** Two control PCRs confirm cDNA synthesis from each sample (single round of PCR, 40 cycles). CD4 PCR also indicates the presence of lymphocytes in each sample. The GAPDH and CD4 PCR products were sequenced and confirmed. An additional PCR (data not shown) was used to establish the absence of genomic DNA from the samples

Table 1 Clinical parameters before and after HSCT

Clinical parameter	Before HSCT (day −60)	After HSCT (day +125)
Serum IgE	5,000 IU/ml	<3 IU/ml
Diarrhea	Severe protracted	Resolved
Feeding requirement	TPN dependent	Exclusively oral feeds
Eczema	Severe	Resolved
Weight	≤3rd percentile for age	10th percentile for age

be considered 'naïve' to Tregs prior to transplant. The rapid Treg populations seen in our patient raises interesting questions regarding Treg development. The emergence of significant numbers of thymic emigrants can take several months, suggesting that the observed Tregs expansion is donor in origin. Presumably, donor-derived mature T cells reconstituted the Treg pool by expanding in vivo. The result produced correction of immune dysregulation, reversal of gut autoimmunity, control of GVHD, and resolution of allergy suggesting donor-origin Tregs have similar functional capacity to endogenous cells.

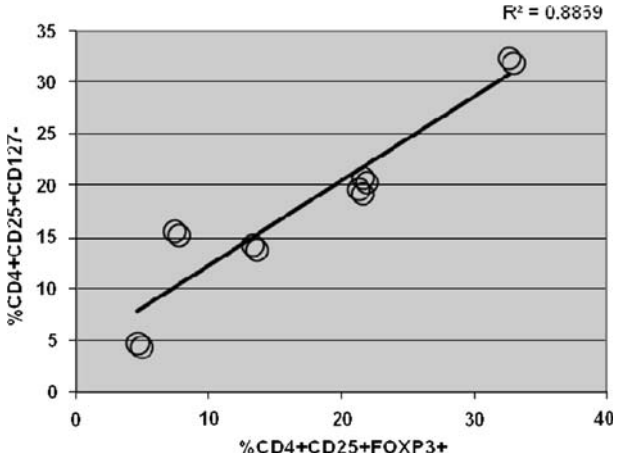

Fig. 3 CD127 as a marker of Tregs following HSCT. Patient's CD4+CD25+FOXP3+ T cells and CD4+CD25+CD127− T cells were examined at six sequential time points following HSCT. Coefficient of determination = 0.8859

Of concern, is whether children with IPEX syndrome can have effective thymopoiesis given absence of FOXP3 expression in thymus stroma which may be essential for the generation of new Treg populations from donor T cell progenitors [8]. Close long-term follow-up will help determine the longevity and functional capacity of Tregs in IPEX syndrome and other primary immunodeficiency diseases.

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