IPEX syndrome is primary immunodeficiency caused by the defects of regulatory T cells (Tregs). IPEX syndrome is often lethal in the first few months of life due to severe diarrhea associated with refractory enteropathy, infections, diabetes mellitus, dermatitis, and other autoimmune complications. This disorder is caused by mutations of Forkhead box protein 3 (*FOXP3*) gene located on chromosome Xp11.23. *FOXP3* encodes Forkhead box protein 3, which is essential for the development and maintenance of CD4+CD25+Foxp3+ Tregs (1, 2).

Established treatments for patients with IPEX syndrome include immunosuppressive therapy and allogeneic HSCT (3–6). Allogeneic HSCT serves as a curative therapy for patients with IPEX syndrome, and RIC regimens have been reported and resulted in better outcome than myeloablative conditioning regimen (7–11). In general, allogeneic HSCT with RIC regimen may increase the risk of rejection and mixed chimera. Some RIC regimens included the antibody against T lymphocytes such as alemtuzumab or ATG. However, these agents may increase the risk of viral reactivation after HSCT. To the best of our knowledge, only two cases of IPEX patients treated with allogeneic HSCT following RIC consisted of low-dose TBI instead of alemtuzumab or ATG have been reported (12).

Here, we report a patient with IPEX syndrome treated with RIC regimen consisted of fludarabine, cyclophosphamide, and low-dose TBI followed by allogeneic HSCT from an HLA-identical sibling donor. Although the patient was in mixed chimera, he was free from symptoms caused by the absence of Tregs. We could observe selective and sustained growth advantage of donor-derived Tregs and disappearance of anti-villin autoantibody in his serum, which was correlated with the improvement in refractory enteropathy.

Patient and methods

Patient

A Japanese male suffered from severe diarrhea at two months of age. He was diagnosed as IPEX syndrome by identifying a missense mutation of T1117G substitution in exon 10 of the *FOXP3* gene (13). We quantified CD4+CD25+Foxp3+ cells by flow cytometry, and positive cells were not identified at all in PBMCs. Autoantibodies examined were negative except anti-villin antibody in patient's serum. He was treated with immunosuppressive therapy of intravenous CyA and oral PSL. After complete remission was achieved, he was free from the symptom for six yr with oral low-dose CyA and PSL (14).

At the age of six, the patient suffered from severe diarrhea again and was referred to our hospital. Although he was treated with increased doses of CyA and PSL in addition to other immunosuppressive agents, these treatments were not effective enough to control his diarrhea completely. We next tried IVIG therapy, which resulted in the improvement in diarrhea, and we could taper immunosuppressive agents.

To control the disease without continuous immunosuppressive therapy, we considered to perform allogeneic BMT from an HLA-matched healthy sibling donor. The donor did not have the mutation in *FOPX3* gene. We used a RIC regimen consisted of 4 Gy (2 × 2 Gy) TBI (day 7), fludarabine at a dose of 30 mg/m^2 for five days (days 6 to day 2) and cyclophosphamide at a dose of 60 mg/kg for two days (days 3 and 2). Total nucleated bone marrow cells of 4.32×10^8/kg were transplanted. We selected CyA and short-term methotrexate as GVHD prophylaxis, and IVIG was continued weekly until autoimmune colitis was resolved.

Chimerism assay

Chimerism assay was performed by polymerase-chain-reaction-based assays analyzing polymorphic short tandem repeat markers (15). The chimerism was examined in each fraction of T cells, total lymphocytes, and granulocytes in bone marrow or peripheral blood. We evaluated the chimerism in bone marrow before day 100 and in peripheral blood after day 100, because we had similar results in both samples before day 100 in the patient and avoided frequent bone marrow aspiration after day 100.

Flow cytometry

PBMCs were stained with monoclonal antibodies of APC-conjugated human CD4, PE-conjugated human CD25, and FITC-conjugated human Foxp3 antibodies (BD Biosciences, San Jose, CA, USA) and analyzed by a FACSCanto II flow cytometer (BD Biosciences), as described previously (16).

Immunoblot analysis of anti-villin antibody

Anti-villin autoantibody in patient's serum was analyzed as described previously (17). Briefly, 500 ng of GST-villin recombinant protein (121 kD) was transferred to the membrane and incubated with diluted serum at 1:160. Anti-villin antibody bound to GST-villin was detected by horseradish peroxidase-conjugated antibody and DAB system.

Case report

Clinical improvement after RIC and allogeneic HSCT

The patient achieved an engraftment on day 11, and the last transfusion of platelets was on day 7 and that of red blood cells was on day 1. He was complicated with transient acute GVHD of the skin (grade I) on day 35 but this resolved without additional immunosuppressive therapy. He had no episodes of significant infection and other severe regimen-related toxicity during the course of RIC and allogeneic HSCT.

Severe and bloody diarrhea settled down on day 14 after engraftment. The patient was

consistently free from symptoms of enteropathy and any other autoimmune diseases. Laboratory findings showed improvement in hypoalbuminemia and anemia caused by severe enteropathy on day 21. Colonoscopy examination on day 60 revealed disappearance of mucosal inflammation, multiple ulcerations and hemorrhage that were observed before the HSCT.

After the discharge on day 120, we had followed the patient every two wk. He had no episodes of autoimmune disorders and infection, and we could taper and stop immunosuppressive agents at six months. Unfortunately, he suffered from MLL gene-rearranged ALL at 24 months after transplantation. The origin of precursor B lymphoblasts was recipient cells. We treated him with chemotherapy and allogeneic PBSCT from the same donor. We used myeloablative conditioning regimen consisted of busulfan at a dose of 4 mg/kg for four days and melphalan at a dose of 90 mg/m^2 for two days for the second transplant from the same sibling donor to cure this secondary ALL. He has been in complete remission for more than two yr. Chimerism completely changed to donor-type and the number of Tregs increased to normal after the second transplant.

Chimerism and immunological evaluation after first allogeneic HSCT

Because the ratio of donor T cells, total lymphocytes, and granulocytes in bone marrow was 74%, 48%, and 48%, respectively, on day 22 after HSCT, we reduced the dose of CyA immediately. The ratio of donor cells, however, was further declined to 5% on day 50, and the donor bone marrow was assumed to be rejected (Fig. 1). At that point, flow cytometric analysis of peripheral blood showed that 17.8% of PBMCs were CD4+CD25+Foxp3+ Tregs (Fig. 2a,b). This discordant result on day 50 was explained by selective expansion of donor-derived Tregs. After discontinuation of CyA on day 50, the ratio of donor T cells, total lymphocytes, and granulocytes was transiently increased up to 40% and then gradually decreased (Fig. 1). At 24 months after HSCT, donor cells were around 20% and CD4+CD25+Foxp3+ Tregs were at the range of 1.2–3.0% of PBMCs, which were comparable to healthy controls (Fig. 2a). We did not perform DLI because the ratio of donor cells was <50% and supposed that the patient was in high risk of bone marrow aplasia after repeated DLI.

The anti-villin autoantibody was detected by immunoblot analysis when the disease was active before HSCT. The antibody was under detectable levels both in clinical remission by immunosuppressive therapy before HSCT and after engraftment was achieved following HSCT even when immunosuppressive agents were not administrated (Table 1).

Discussion

The defect of Tregs in patients with IPEX syndrome causes symptoms related to

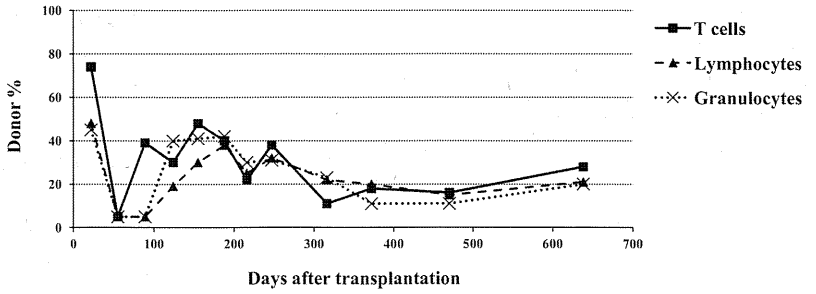

Fig. 1. Frequency of donor cells after allogeneic HSCT. Percentages of donor cells in each fraction of T cells, total lymphocytes, and granulocytes after HSCT are shown.

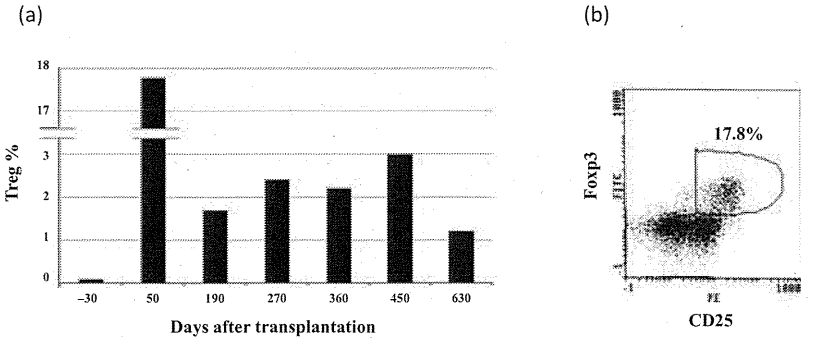

Fig. 2. Sustained expansion of donor Tregs after allogeneic HSCT. (a) Percentages of Tregs are evaluated as CD4+CD25+Foxp3+ cells in PBMCs. (b) Flow cytometric demonstration of CD25+Foxp3+ cells in CD4 gated PBMCs on day 50 after HSCT.

Table 1. Anti-villin autoantibody is correlated with clinical condition of enteropathy

Clinical condition of enteropathy	Anti-villin autoantibody
Disease onset	+
Remission before HSCT	−
Relapse before HSCT	+
After HSCT	−

GST-villin protein (121 kD) was transferred to the membrane, and immunoblot was performed with 1:160 diluted patient's serum at different disease condition of enteropathy as indicated. + indicates the presence of anti-villin antibody that recognizes GST-villin protein.

autoimmunity. However, clinical benefit of immunosuppressive therapy is often limited by its adverse effects and increased susceptibility to infection. At present, allogeneic HSCT is recognized as the curative therapy for patients with IPEX syndrome. We summarized all reported cases treated with HSCT in Table 2. Myeloablative regimen resulted in high fatality due to regimen-related toxicity or lethal infection (2, 5, 6). On the other hand, Rao et al. (7) first reported four patients who were successfully treated with non-myeloablative conditioning regimen consisted of fludarabine, melphalan, and alemtuzumab, and achieved high rate of donor chimerism above 84.6%. Non-myeloablative regimens with anti-T-lymphocyte antibody such as alemtuzumab or ATG have been used, and all patients are alive (7–11). However, it is known that alemtuzumab and ATG induce profound depletion of T cells and increase the risk of viral reactivation and fungal infection after HSCT. Therefore, we used low-dose TBI instead of anti-T-lymphocyte antibodies. The combination of low-dose TBI, fludarabine, and cyclophosphamide was well tolerated, and the patient was free from infections and severe regimen-related toxicities. Burroughs et al. (12) also reported that RIC regimen including low-dose TBI for IPEX syndrome resulted in stable engraftment of Tregs and better clinical outcome, proposing that this regimen was preferable for patients with IPEX syndrome.

The patient developed MLL-related secondary ALL in recipient cells. Although the dose of TBI was less than used in myeloablative conditioning, radiation and alkylating agents might cause DNA damage and increased the risk of secondary leukemia in recipient cells. Alternatively, the use of anti-T-lymphocyte antibody instead of low-dose TBI and/or dose reduction in alkylating agents should be carefully considered in IPEX syndrome.

Selected and sustained expansion of Tregs resulted in clinical improvement even though the patient was in mixed chimera after HSCT. Seidel et al. (11) reported a patient with IPEX

Table 2. Summary of IPEX patients treated with allogeneic HSCT reported in the literature

Case	Age	Donor	Conditioning regimen	Complications after HSCT	Outcome	% Donor after HSCT	Reference
1	13 yr	HLA-matched sibling	TBI 12 Gy + CY + ATG	Adenovirus infection, pneumonia	Dead	50%	2
2	9 yr	HLA-matched unrelated	TBI 12 Gy + CY + ATG	Cytomegalovirus infection, hemorrhagic cystitis, lymphoproliferative disorder	Dead	70%	2
3	4 months	HLA-matched sibling	BU + CY + ALG	Hemophagocytic syndrome	Dead	30% in T cell	5
4	1 yr	HLA-matched sibling	BU + CY + Flu + ATG		Alive	70% in T cell	6
5	7 yr	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Cytomegalovirus infection	Alive	100%	7
6	1 yr	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Acute respiratory distress syndrome	Alive	100%	7
7	4 yr	HLA-matched sibling	Flu + L-PAM + alemtuzumab	Histoplasma infection	Alive	89%	7
8	5 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab		Alive	84.6%	7
9	7 yr	HLA 5/6-matched cord blood	Flu + BU + ATG	Lymphoproliferative disorder	Alive	81 ~ 98%	8
10	7 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Sepsis of *Enterobacter cloacae*	Alive	100%	9
11	5 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab + anti-CD 45 monoclonal antibody		Alive	100%	10
12	11 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab		Alive	<10%	11
13	9 months	HLA-matched unrelated	TBI 4 Gy + Flu	Bacteremia	Alive	100%	12
14	16 yr	HLA-matched related	TBI 4 Gy + Flu	Bacteremia	Alive	20 ~ 60% in T cell	12

Case series transplanted with RIC regimens were highlighted.
CY, cyclophosphamide; ALG, antilymphocyte globulin; BU, busulfan; Flu, fludarabine; L-PAM, melphalan.

syndrome who showed selective engraftment of Tregs for six yr after non-myeloablative transplantation. It has been reported that partial BMT or injection of T-enriched splenocytes resulted in the rescue of autoimmunity in Scurfy mice, a mouse model for IPEX syndrome in which *FOXP3* gene is naturally mutated. Sustained engraftment of relatively high frequency of CD4+CD25+Foxp3+ Tregs was observed even though the frequency of donor cells in whole peripheral blood ranged from 1.7% to 50% (18). These observations illustrate that the paradigm in the generation of Tregs is reinforced by the requirement and growth advantage regardless of chimerism of other hematopoietic cells in IPEX syndrome. However, we should still consider the possibility that mixed chimerism may result in subsequent development of autoimmune diseases observed in other primary immunodeficiency, as previously reported in some patients with Wiskott–Aldrich syndrome (19).

Intractable diarrhea is a major symptom in patients with IPEX syndrome. Villin, an actin-binding protein, is expressed as the 95 kD antigen in the small intestine, which is frequently targeted by autoantibodies in patients with IPEX syndrome (17). Anti-villin antibody was clearly correlated with the severity of clinical symptoms in our patient. Therefore, monitoring of anti-villin antibody might serve as a useful examination for evaluating gastrointestinal complications in patients with IPEX syndrome.

We reported here a unique phenomenon of selective growth advantage of Tregs in a patient with IPEX syndrome who was in mixed chimera after RIC and allogeneic HSCT. Sustained expansion of donor-derived Tregs resulted in the significant improvement in enteropathy. To determine optimal RIC regimen to achieve complete chimera and avoid secondary malignancy in residual recipient cells, further analysis in more patients and long-term follow-up study after HSCT are required to conclude this issue.

Authors' contributions

Horino S and Sasahara Y designed the study, interpreted the data, wrote the paper, and treated the patient. Sato M, Kanegane H, Kamachi Y, and Kobayashi I performed experiments. All other authors treated the patient and collected clinical data.

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Conflict of interest disclosure

The authors declare no conflict of interest.

References

1. Powell BR, Bruist NR, Stenzel P. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. J Pediatr 1982: 100: 731–737.
2. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. J Med Genet 2002: 39: 537–545.
3. Taddio A, Faleschini E, Valencic E, et al. Medium-term survival without hematopoietic stem cell transplantation in a case of IPEX: Insights into nutritional and immunosuppressive therapy. Eur J Pediatr 2007: 166: 1195–1197.
4. Gambineri E, Perroni L, Passerini L, et al. Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: Inconsistent correlation between forkhead box protein 3 expression and disease severity. J Allergy Clin Immunol 2008: 122: 1105–1112.
5. Baud O, Goulet O, Canioni D, et al. Treatment of the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) by allogeneic bone marrow transplantation. N Engl J Med 2001: 344: 1758–1762.
6. Mazzolari E, Forino C, Fontana M, et al. A new case of IPEX receiving bone marrow transplantation. Bone Marrow Transplant 2005: 35: 1033–1034.
7. Rao A, Kamani N, Filopovich A, et al. Successful bone marrow transplantation for IPEX syndrome after reduced-intensity conditioning. Blood 2007: 109: 383–385.
8. Lucas KG, Ungar D, Comito M, et al. Submyeloablative cord blood transplantation corrects clinical defects seen in IPEX syndrome. Bone Marrow Transplant 2007: 39: 55–56.
9. Dorsey MJ, Petrovic A, Morrow MR, et al. FOXP3 expression following bone marrow transplantation for IPEX syndrome after reduced-intensity conditioning. Immunol Res 2009: 44: 179–184.
10. Zhan H, Sinclair J, Adams S, et al. Immune reconstitution and recovery of FOXP3 (forkhead box protein 3)-expressing T cells after transplantation for IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome. Pediatrics 2008: 121: 998–1002.
11. Seidel MG, Fritsch G, Lion T, et al. Selective engraftment of donor $CD4^+CD25^{high}$ FOXP3-positive T cells in IPEX syndrome after nonmyeloablative hematopoietic stem cell transplantation. Blood 2009: 113: 5689–5691.
12. Burroughs LM, Torgerson TR, Storb R, et al. Stable hematopoietic cell engraftment after low-intensity nonmyeloablative conditioning in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. J Allergy Clin Immunol 2010: 126: 1000–1005.
13. Tanaka H, Suzuki K, Nakahata T, et al. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Acta Paediatr 2004: 93: 142–143.
14. Tanaka H, Tsugawa K, Kudo M, et al. Low-dose cyclosporine A in a patient with X-linked immune dysregulation, polyendocrinopathy and enteropathy. Eur J Pediatr 2005: 164: 779–780.

15. Thiede C, Florek M, Bornhäuser M, et al. Rapid quantification of mixed chimerism using multiplex amplification of short tandem repeat markers and fluorescence detection. Bone Marrow Transplant 1999: 23: 1055–1060.
16. Otsubo K, Kanegane H, Kamachi Y, et al. Identification of FOXP3-negative regulatory T-like ($CD4^+CD25^+CD127^{low}$) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Clin Immunol 2011: 141: 111–120.
17. Kobayashi I, Kubota M, Yamada M, et al. Autoantibodies to villin occur frequently in IPEX, a severe immune dysregulation, syndrome caused by mutation of FOXP3. Clin Immunol 2011: 141: 83–89.
18. Smyk-Pearson SK, Bakke AC, Held PK, et al. Rescue of the autoimmune scurfy mouse by partial bone marrow transplantation or injection with T-enriched splenocytes. Clin Exp Immunol 2003: 133: 193–199.
19. Ozsahin H, Cavazzana-Calvo M, Notarangelo LD, et al. Long-term outcome following hematopoietic stem-cell transplantation in Wiskott-Aldrich syndrome: Collaborative study of the European Society for Immunodeficiencies and European Group for Blood and Marrow Transplantation. Blood 2008: 111: 439–445.

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