Quantitative analysis of tissue inflammation and responses to treatment in immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, and review of literature

Chih-An Chen, Wan-Chen Chung, Yuan-Yow Chiou, Yao-Jong Yang, Yung-Chieh Lin, Hans D. Ochs, Chi-Chang Shieh

Department of Pediatrics, National Cheng Kung University Hospital, Tainan, Taiwan
Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
Seattle Children’s Research Institute and Department of Pediatrics, University of Washington, Seattle, WA, USA

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Abstract  Background/Purpose: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a severe autoimmune disease that is caused by regulatory T cell deficiency due to FOXP3 gene mutations. The long-term outcome can be variable depending on the extent of tissue damage caused by autoimmunity and infections, the use of immunosuppressive treatment or sequela of bone marrow transplantation.  Methods: We used immunohistochemical staining to analyze cell types infiltrating the tissue of affected organs from a classic IPEX patient with a splicing mutation (c.736-2A>C) in the FOXP3 gene. Expression of transcription factors that are critical for immune responses including T-bet, GATA-3, RORγt, and FOXP3 were evaluated in various tissue samples. For objective analysis of the distribution of different cell types in tissues, we used an automated microscope-based image acquiring system to assess quantitatively the different cell types by investigating the histopathological changes in the patient’s biopsy samples obtained from the intestine and the kidneys before and after treatment.  Results: The percentages of cells expressing the T_{H2}-associated transcription factor GATA3 were higher in the IPEX patient before treatment than in controls, suggesting that T_{H2}-type...
Introduction

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a life-threatening disease that was first described in 1982. It usually presents in young infants or children and leads to severe, multiorgan autoimmune phenomena including chronic enteropathy, dermatitis, and endocrinopathy, such as type I diabetes mellitus and thyroiditis. Other abnormalities frequently observed include autoimmune cytopenia (i.e., hemolytic anemia, thrombocytopenia, or neutropenia), nephritis, hepatitis, and elevated levels of serum immunoglobulin (Ig) A and E. Episodes of severe infections are often reported in patients with this syndrome. IgG and IgM levels are typically normal but can be slightly depressed in older individuals, probably as a result of enteric protein loss. It is a relatively rare disorder with fewer than 160 cases described worldwide over a 10-year period. Without appropriate treatment, this syndrome is fatal in most affected children within the first 2 years of life. Germ-line mutations in the FOXP3 gene, a master transcriptional regulator for the development of CD4+CD25+CD127low regulatory T cells (Treg), were found in many male patients with the IPEX phenotype.

Tregs, a T cell subpopulation with broad inhibitory immune functions, are important for maintaining self-tolerance and immune homeostasis. The FOXP3 gene encodes the forkhead box protein 3, a member of the forkhead/winged-helix family of DNA-binding transcriptional regulators, which is essential for Treg differentiation. In humans, the FOXP3 gene is located on the X chromosome at Xp11.23. Although FOXP3 gene mutations leading to loss of function have been associated with the syndrome, the exact immune mechanisms leading to tissue damage in IPEX syndrome patients have rarely been investigated. In this study, we aimed to measure quantitatively the inflammatory cell infiltrates in critical internal organs damaged by autoimmunity in a patient with classic IPEX syndrome, and to determine the dominant immune effector cells in the tissues damaged by the disease.

Case Report

The index case was the second child of nonconsanguineous parents. The boy was born via cesarean delivery due to breech presentation with a birth weight of 2950 g and length of 51 cm. Since the age of 2 months, generalized skin scaling was present (Figure 1A), he was repeatedly hospitalized for pneumonia due to Staphylococcus aureus, and chronic diarrhea leading to severe malnutrition and failure to thrive. Endoscopy revealed a pale and fragile mucosa, affecting the entire upper and lower gastrointestinal tract. Histological analysis of gastrointestinal tract biopsies demonstrated extensive villous atrophy with widespread leukocyte infiltration. The lamina propria of the colonic mucosa was infiltrated by lymphocytes, plasma cells, eosinophils, and neutrophils. Laboratory evaluation revealed elevated IgE levels (up to 18,000 IU/mL) but normal IgG, IgM, and IgA. Hypoalbuminemia, possibly related to protein-losing enteropathy, was persistently observed. Immunological analysis using flow cytfluorometry performed at the onset of diarrhea showed a normal distribution of lymphocyte subpopulations (CD3: 2829/μL; CD4: 1511/μL; CD8: 1085/μL; and CD19: 814/μL HLA-DR: 1744/μL). In his early infancy, the patient suffered from multiple episodes of bacterial and candida sepsis and pruritic eczematous dermatitis. At age 1 year, type I diabetes mellitus was diagnosed. Genomic DNA sequencing of the FOXP3 gene revealed a splice-site mutation upstream of exon 7 (c.736-2 A>C). Sequence analysis of the FOXP3 cDNA demonstrated skipping of exon 7, which causes an in-frame deletion of 27 amino acids upstream of the forkhead domain. The same heterozygous mutation was found in the mother. We started calcineurin inhibitor [i.e., cyclosporine A and tacrolimus (FK506)] treatment at the age of 2 years and 3 months resulting in improvement of the skin lesions (Figure 1B), diarrhea, and growth retardation, and total parenteral nutrition could be discontinued. Serial histological studies revealed a gradual improvement of the inflammatory lesions throughout his digestive tract. However, renal tubular acidosis was noted at age 2 1/2 years. Nephrotic syndrome due to membranous glomerulonephritis developed at age 7 years and 10 months in an attempt to control the glomerulonephritis, with clinical improvement. Hematopoietic stem cell transplantation was refused by the family. The patient died at age 8 years and 4 months of Streptococcus pneumoniae sepsis.

Histological examination

Blocks of paraffin-embedded intestinal biopsies from the IPEX patient and normal controls were provided by the Department of Pathology, National Cheng Kung University.
Hospital (NCKUH), Tainan, Taiwan. Intestinal tissue biopsies with normal gross and microscopic appearance from a patient who was evaluated for allergic intestinal inflammation were used as comparative controls. The renal biopsy was fixed in 10% buffered neutral formalin and embedded in paraffin. Tissue sections (2-3 μm) were cut onto Dako slides (Dako-Cytomation, Glostrup, Denmark), incubated at 45°C overnight, and deparaffinized three times using xylene (5 minutes, room temperature). The slides were rehydrated in a series of ethanol solutions (100% and 95% for 2 minutes each) and washed with Tris-buffered saline and tween (TBST) 20, then stained with hematoxylin and eosin using standard procedures.

Immunohistochemistry

For immunohistochemistry staining, tissue sections from the IPEX patient and a normal control, cut on poly-L-lysine-coated slides, were deparaffinized in xylene and rehydrated in a series of ethanol. Antigen retrieval was performed by microwave cooking (Sampo, 800W output) for 2 minutes in 10mM citrate buffer (pH 6.0), and then cooled to room temperature for 30 minutes and washed with TBST once. The endogenous peroxidase activity was blocked with PolyDetector Peroxidase Block solution (Bio SB, Goleta, CA, USA) for 30 minutes at room temperature then washed three times with TBST. Following immune blocking with 1% bovine serum albumin for 30 minutes, the slides were incubated with primary antibodies overnight, and the signals visualized by immunohistochemical procedures.

Quantitative measurement of transcription factors involved in T cell development

We used immunohistochemistry and objective quantification to detect the different types of T helper cells in the intestinal and kidney biopsies of the IPEX patient and of a control with allergic intestinal inflammation.

For objective counting of different cell types in tissues, we used a microscope-based image acquiring system that can create large overviews of tissue specimen with high magnification (TissueGnostics Cytometry; TissueGnostics GmbH, Vienna, Austria) to scan the slides with high magnification. We scanned the whole biopsy tissue and selected at least four representative areas to analyze the proportion of positive stained cells to avoid the observational bias. We used computer-assisted analysis Histoquest software (TissueGnostics GmbH), which enhanced the accuracy and objectiveness in histopathology to study the expression of multiple transcription factors known to play important roles in T cell development, using the following antibodies for tissue staining according to the respective manufacturers’ instructions: H-210: sc-21003 (Santa Cruz Biotechnology, Dallas, TX, USA) for T-bet, 10417-1-AP (Proteintech Group, Chicago, IL, USA) for GATA-
3, Ab78007 (Abcam, Cambridge, UK) for RORγt and 14-4776 (eBioscience, San Diego, CA, USA) for FOXP3.

Statistical analysis

The expression level of T-bet, GATA-3, RORγt, and FOXP3 in tissues were statistically analyzed with Student t test using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA). A p value < 0.05 was considered statistically significant.

Results

Characterization of Treg deficiency

Flow cytometry data demonstrated that the IPEX patient had few CD4+ expressing FOXP3+ lymphocytes (0.3%) in the peripheral blood when compared with a normal control (2.7%; Figure 1C). Immunohistochemical staining was carried out to examine the percentage of FOXP3-positive lymphocytes in the intestinal tissue of the IPEX patient in comparison with a control who had nonspecific intestinal inflammation. The intestinal biopsy sections from the IPEX patient contained no FOXP3+ lymphocytes while, in the control with nonspecific intestinal inflammation, we could easily identify many FOXP3+ (Figure 2A). Quantitative analysis showed that the IPEX patient expressed < 0.3% FOXP3+ cells compared with 3% in the tissue from the control with nonspecific intestinal inflammation (Figure 2B).

T cell specific transcription factors expression in the intestinal mucosa

We then used immunohistochemical staining to quantify the key transcription factors for T cell differentiation in the intestine of our IPEX patient before and after calcineurin inhibitor FK506 (tacrolimus) treatment. After treatment, the percentage of T-bet-expressing lymphocytes was significantly (p < 0.001) reduced (Figure 3). The Th2 cell transcription factor GATA-3 was expressed at a higher percentage in gut tissue of the IPEX patient than in the control patient before treatment. However, after treatment with FK506, the percentage of GATA-3 expressing lymphocytes markedly decreased, reaching levels that were even lower than those of the control (Figure 3). The percentage of RORγt expressing lymphocytes was slightly higher than that in the control before the calcineurin inhibitor treatment, but showed no significant change after FK506 treatment (Figure 3).

Histological examination of renal biopsy sections before and after immunomodulatory treatment

As the kidneys are frequently affected in patients with IPEX (as was the case in our patient), we investigated the immunopathology of the renal tissue to elucidate the autoimmune-mediated tissue damage. Electron microscopy revealed diffuse thickening of the glomerular basement membrane and a few tubular reticular bodies in endothelial cells representing electron dense immune

Figure 2. Markedly reduced FOXP3-positive lymphocytes in the blood and the intestine of a patient with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. (A) Immunohistochemistry staining for FOXP3. The IPEX patient shows no FOXP3 positive lymphocytes in the intestinal section, while the control has FOXP3 positive cells in the small bowel mucosa. Red arrows, FOXP3-positive cells have a dark nucleus. The yellow arrows indicate unstained lymphocytes (original magnification 400×). (B) Quantitative analysis of FOXP3 positive cells in the small bowel. The control with nonspecific inflammation has 3% of FOXP3 positive lymphocytes while the patient has < 0.3%.
complexes (Figure 4A). The hematoxylin and eosin staining shows focal segmental expansion of mesangial matrix with mesangial hypercellularity (Figure 4A). Because anti-CD20 monoclonal antibody (Rituximab) improved the nephritis of the IPEX patient, we analyzed the key transcription factors for T helper cell differentiation in the kidney of the IPEX patient before and after Rituximab treatment (Figure 4B). Quantification of the positively stained lymphocytes in the glomeruli of renal biopsy showed that before treatment, GATA-3-expressing lymphocytes were the major infiltrating cells, being more frequent than T-bet or RORγt-expressing lymphocytes (Figure 4C). After Rituximab treatment, the percentage of GATA-3 and RORγt-expressing lymphocytes decreased significantly (p < 0.05), while the percentage of T-bet-expressing lymphocytes did not differ significantly in pre- and post-treatment collected samples.

Discussion

Regulatory T cells are indispensable for maintaining self-tolerance and immune homeostasis. Treg deficiency has been attributed to the pathogenesis of many autoimmune diseases, including IPEX syndrome, type I diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus. In this study, we evaluated a patient with a splicing mutation in the FOXP3 gene leading to severe Treg deficiency who presented as typical IPEX syndrome. We found that the expression of the Th2 cell-associated transcription factor GATA3 in the IPEX patient was higher than in the control before treatment with calcineurin inhibitor, and decreasing after treatment. Th1-associated transcription factor T-bet expression in the IPEX patient was lower than control before and after treatment. Th17-associated transcription factor RORγt expression in the IPEX patient was also higher than in the control but showed no difference when measured before and after treatment.
decreased significantly after effective immunosuppressive therapy. These observations suggest that TH2-type cells are important effector cells responsible for immune-mediated damage of the intestine and kidneys in IPEX syndrome. The IPEX syndrome is caused by mutations in the FOXP3 gene that is located on the X chromosome (Xq11.23) and consists of 11 coding exons (exons 1–11) and three non-coding exons.6,15 Phenotypically, most patients with causative FOXP3 mutations have classic IPEX syndrome. To date, > 30 unique FOXP3 mutations have been identified in patients with IPEX, mainly clustering at the beginning and in the third part of the gene—most of which result in a change in the amino acid sequence in the DNA-binding forkhead domain of the FOXP3 protein.7,16,17 Our patient’s genomic DNA sequencing of the FOXP3 gene revealed a novel A > C mutation in the invariant splice site upstream of exon 7 (c.736-2 A > C) resulting in skipping of exon 7 and the in-frame deletion of 27 amino acids upstream of the DNA binding domain, which encodes part of the leucine zipper domain and is expected to interfere with

Figure 4. Changes in the concentration of the T helper cell-associated transcription factor expression suggest that TH2-type and TH17-type cells may participate in the tissue inflammation of immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome nephritis. (A) Electron microscopy shows a few electron-dense depositions in mesangial areas with thickening of glomerular basement membrane (left panel), and a few tubulo-reticular bodies (arrows) in endothelial cells (central panel). The hematoxylin and eosin stain shows segmental expansion of mesangial matrix (right panel). (B) Immunohistochemical staining (brown staining of nuclei) for T-bet, GATA-3, and RORγt are detected within glomeruli in the renal biopsies of the IPEX patient before and after treatment with Rituximab. (C) Quantitative assessment of glomerular cells in the renal sections described in (B). The percentage of cells expressing GATA-3 was higher than that of T-bet and RORγt, and decreased significantly (p < 0.002) after treatment. The expression of T-bet did not differ before and after treatment. The percentage of RORγt expressing cells within glomeruli also decreased significantly (p < 0.0001) after treatment.
protein homodimerization and its transcriptional activity. Although we did not directly measure the stability of the produced protein in this study, the absence of FOXP3 protein in the patient’s lymphocytes suggests that the truncated protein is unstable.

Previous descriptions of immune-mediated tissue damage in IPEX patients focused mainly on endocrine organs and the intestinal tract. It was suggested that T\(_{H1}\) and T\(_{H17}\) cells functioned as mediators of tissue damage and autoimmunity leading to immune dysregulation and lymphocytic infiltrates of major organ systems characteristic for IPEX. In addition, Tregs have been shown to be important for the maintenance of peripheral B-cell tolerance in humans. However, almost one-third of IPEX patients have renal involvement with histological changes ranging from immune complex deposition to interstitial nephritis.

It is unclear whether the renal manifestations are secondary to the treatment, which often include drugs with renal toxicity or are related to the disease itself. However, IPEX patients with renal disease prior to the use of immunosuppressive drugs had been reported. It has been reported that IPEX patients who underwent successful bone marrow transplants showed rapid improvement of their renal disease with normalization of renal function while the outcome in patients treated only with calcineurin inhibitors is less favorable. In this study, we found that before treatment, T\(_{H2}\)-type cells were the major infiltrating cells in the glomeruli, with a higher proportion than that of T\(_{H1}\) and T\(_{H17}\) cells. Interestingly, after treatment with Rituximab both T\(_{H2}\)-type and T\(_{H17}\)-type cells significantly decreased while the proportion of T\(_{H1}\) cells did not change significantly, suggesting a role of auto-antibody in the pathogenesis of renal disease in IPEX.

The clinical presentation of IPEX, which includes elevated serum IgE and dermatitis lesions similar to atopic dermatitis, implicates the role of T\(_{H2}\)-type cells in the disease. T helper cells have been demonstrated to be critical for B cell activation and antibody production. In mice, T\(_{H1}\) cells preferentially induce IgG2a, while T\(_{H2}\) cells induce IgG1 and IgE secretion. Our findings that T\(_{H2}\)-type cells are the major effector cells in the intestine and the kidneys suggest that autoantibody production might be among the important mechanisms of autoimmune tissue damage. The presence of tubule-reticular inclusion bodies, a characteristic finding in lupus nephritis, in the kidney biopsies from patients with IPEX suggests that autoantibody production might be among the major effector cells in the intestine and the kidneys.

Three primary immunodeficiency diseases (IPEX, Wiskott–Aldrich syndrome, and Omenn syndrome) are reported to be associated with high levels of IgE and defective Treg cell number or function. The uncontrolled TH2 type immune responses not only dominate the autoimmune responses in the target tissues but also hinder the host from mounting effective immune responses to invading microorganisms requiring other types of immune defense. A shared deregulation of immune responses may lead to the defective immunity in these primary immunodeficiency diseases with defective Treg functions.

Glucocorticoid monotherapy or combination immunosuppression has been shown to be only partially effective in controlling the autoimmune manifestations. Although they are used as the first line therapy to limit progression of organ damage, glucocorticoids complicate diabetes management and are associated with significant side effects with long-term use in children. Other immunosuppressive drugs thus are added onto the steroids regimens. Cyclosporine and/or tacrolimus, as used in this report, have been most commonly used in conjunction with steroids.

Like T helper cells, subpopulations of innate lymphoid cells (ILCs) have been shown to react promptly to signals from infection or tissue damage and produce cytokines to direct the development of autoimmune responses. A number of transcription factors including T-bet, GATA-3, and RORγt have been shown to be expressed in ILC subpopulation and are important for the development of ILCs. It is likely that the T-bet, GATA-3, and RORγt-expressing lymphocytes we identified in this study (Figures 3 and 4) include both T cells and ILCs. Although FOXP3 has not been shown to be expressed in ILCs, lack of FOXP3 and hence Tregs may well affect the activity of ILCs and lead to immune-mediated tissue damage. A recent report demonstrated that FOXP3+ Tregs can directly inhibit a subpopulation of ILCs and thereby suppress innate cell-driven colitis. If and how ILCs participate in the pathogenesis of IPEX syndrome merits further investigation.

In the clinical setting, histological examinations are important for the understanding of the pathogenic processes and for choosing appropriate treatments. Most histologic techniques for quantitatively assess pathologic events in histology studies randomly select areas in the tissue and use a grading system that is more or less subjective. Hence, the results heavily depend on the observer who uses their own standard of quantitative measurement. In this study, we selected an automated microscope-based image acquiring system that can create large analysis of tissue specimen with high resolution. The software we used automatically quantifies the staining intensity of signals in the tissue. This system solved the problem to quantitatively assess effector cells in the tissue of our IPEX patient. Thus we were able to identify critical immune-pathogenic changes that may well have been overlooked otherwise.

In conclusion, this study provides direct evidence that the autoinflammation affecting the GI tract and kidneys in our IPEX patient is caused by T\(_{H2}\)-dominant effector mechanisms. After immunosuppressive therapy with tacrolimus and Rituximab or with Rituximab alone, the T\(_{H2}\)-type lymphocyte infiltration was suppressed and the autoimmune tissue damage in the GI tract and the kidneys improved. To our best knowledge this is the first direct evidence that T\(_{H2}\)-type lymphocytes are the major cells infiltrating the affected tissues from IPEX patients. We speculate that compounds targeting T\(_{H2}\)-type cells may be an effective therapy to target autoimmune-mediated internal organ damage of patients with IPEX.

Conflicts of interest

All contributing authors declare no conflicts of interest.
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