Molecular mechanisms in allergy and clinical immunology
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Immune dysregulation, polyendocrinopathy, enteropathy, X-linked: Forkhead box protein 3 mutations and lack of regulatory T cells

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The rare X-linked disorder immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) and its murine counterpart scurfy have provided important new insights into the essential role of regulatory T cells (Treg) in maintaining tolerance to self-antigens. Mutations of the *FOXP3* gene, identified in patients with IPEX, have helped pinpoint key structural domains of the protein that are essential for its function as a transcriptional regulator. Ongoing work using these and associated models has begun to elucidate factors important for the development, function, and competitive fitness of Treg. This improved understanding is beginning to lead to the identification of other defects that may be present in patients who have the clinical phenotype of IPEX but only wild-type FOXP3. It has also led to improved treatment options for IPEX including immunosuppressive drugs and bone marrow transplantation. We are hopeful that the knowledge gained about mechanisms that regulate FOXP3 expression and Treg function will have a major effect on how other autoimmune and allergic disorders are approached. (J Allergy Clin Immunol 2007;120:744-50.)

Key words: *Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX), IPEX-like, autoimmunity, FOXP3, transcription factor, regulatory T cells, scurfy mice, immunosuppressive therapy, stem cell transplantation*

Abbreviations used

FOX:	Forkhead box
FOXP3:	Forkhead box protein 3
IL-2Rα:	IL-2 receptor α
IPEX:	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
PIDD:	Primary immune deficiency disorder
Treg:	Regulatory T cell

The adaptive immune system is designed to protect the host from overwhelming bacterial, fungal, parasitic, and viral infections without accidentally attacking the host and inducing autoimmune disorders. When adaptive immune responses are defective as in patients with primary immune deficiency disorders (PIDDs), there is frequently a breakdown of self-tolerance mechanisms as well. This is evidenced by the high rate of autoimmunity associated with some PIDDs. In addition, a new class of PIDD has recently been characterized in which the primary immune defects interfere with tolerance induction and regulatory mechanisms affecting the immune system. These 3 distinct clinical entities, each caused by a single gene defect, have been associated with multiple autoimmune disorders: the autoimmune lymphoproliferative syndrome (ALPS), characterized by lymphadenopathy, splenomegaly, autoimmune hemolytic anemia, thrombocytopenia, neutropenia, and other autoimmune manifestations is caused by mutations in 1 of several genes involved in apoptosis.[1] The 2 other syndromes associated with multiple autoimmune disorders are both caused by mutations of transcriptional regulatory proteins. Autoimmune polyendrocrinopathy candidiasis ectodermal dystrophy (APECED) is caused by mutations in the autoimmune regulator (*AIRE*) gene,[2] and immune dysregulation, polyendrocrinopathy, enteropathy, X-linked (IPEX) is caused by mutations in the forkhead box protein 3 (*FOXP3*) gene.[3] Each of these disorders has yielded new insights into mechanisms of tolerance induction and maintenance in human beings, but studies surrounding IPEX and its murine homolog scurfy have been particularly fruitful over the past 6 years. In this review, we focus on the spectrum of clinical symptoms and laboratory findings in patients with IPEX and IPEX-like syndromes and describe the role of FOXP3 in the generation of regulatory T cells (Treg).

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IPEX

IPEX phenotype

The clinical entity that has become known as IPEX (Online Mendelian Inheritance in Man 304930) was described 25 years ago by Powell et al[4] as a syndrome of enteropathy, endocrinopathy, and fatal infections affecting young male patients. The presence of this phenotype in multiple generations of the same kindred suggested that the disorder was genetic and followed an X-linked pattern of inheritance.[4] The presence of autoantibodies, Coombs-positive anemia, lymphocytic infiltrates in the pancreas and thyroid, and thymic involution in various patients in this kindred led the authors to postulate that the disorder was immunologically mediated, although they state that it did not have ''the hallmarks of any immunologic disorder previously described.''[4] With the identification of more patients with IPEX, it is now recognized that most patients present with a basic clinical triad of enteropathy, endocrinopathy (diabetes or thyroid disease), and dermatitis. They also frequently have other associated autoimmune disorders.

The gastrointestinal disease of IPEX is often the presenting symptom of the disorder and is characterized by severe villous atrophy and extensive lymphocytic infiltrates of the bowel mucosa (Fig 1, *A*). Clinically, this results in severe watery diarrhea that is at times mucoid or bloody. The diarrhea may markedly worsen after an affected infant is switched from being breast-fed to regular formula, and some patients develop severe food allergies.[5] Symptoms may or may not respond to dietary manipulation and consequently, patients often require total parenteral nutrition to reverse severe failure to thrive. Immunosuppressive therapy often improves but may not resolve the gastrointestinal symptoms.

Early-onset autoimmune endocrinopathies involving the pancreas or thyroid are a characteristic feature of IPEX syndrome. Insulin-dependent type 1 diabetes is the most common endocrine disorder, frequently beginning during the first year of life. In some cases, glucose intolerance is present at birth. Patients who have diabetes often have anti–islet cell antibodies as well as chronic interstitial inflammation and lymphocytic infiltrates in the pancreas (Fig 1, *B*) with complete destruction of islet cells.[6] Thyroiditis is common and can lead to either a hypothyroid or hyperthyroid state. Hypothyroidism occurs most frequently and is associated with elevated thyroid-stimulating hormone levels and/or antithyroid microsomal antibodies.[4,7-9] Interestingly, autoimmune involvement of other endocrine organs such as parathyroids or adrenals is rare in IPEX.

The third component of the IPEX triad is dermatitis, which is most commonly reported to be a mild or moderate eczematous rash. Some infants present with an erythematous rash involving the entire body. Other immune-mediated rashes have recently been described in older patients with IPEX including a psoriasiform dermatitis in an 11-year-old and pemphigoid nodularis in a 14-year-old.[7,10] Alopecia universalis has been observed in several affected patients.[7] Histologically, many of the skin lesions of IPEX are characterized by lymphocytic infiltrates[7] and may improve with topical steroid and immunomodulatory agents.

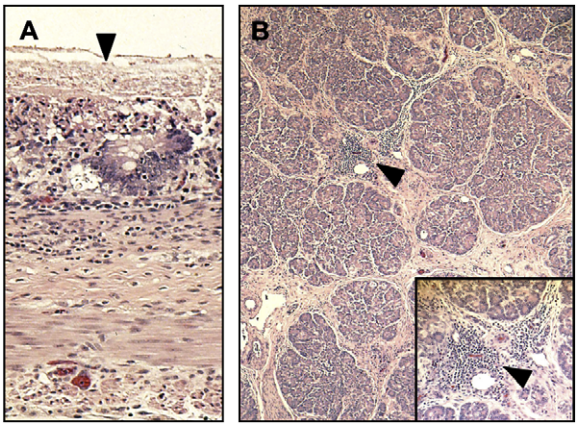

FIG 1. Bowel and pancreas pathology in IPEX. Hematoxylin and eosin stains of small bowel **(A)** and pancreas **(B)** from a patient with IPEX syndrome. Note the complete villous atrophy of the bowel mucosa *(arrow)* as well as marked lymphocytic infiltrate in the intestinal wall. Note the lack of pancreatic islets, diffuse fibrosis, and marked lymphocytic infiltrates in the pancreas (shown at higher magnification in the *inset*).

In addition to the basic IPEX triad, patients with FOXP3 mutations typically have other autoimmune phenomena as well. The most common of these are autoimmune hematologic disorders including Coombs-positive hemolytic anemia, autoimmune thrombocytopenia, and autoimmune neutropenia, which together affect approximately 50% of patients.[3,4,6,7] Relevant autoantibodies can frequently be demonstrated in the serum.

Renal disease is also quite common, affecting approximately 1/3 of patients. It can range in severity from mild hematuria and proteinuria to rapidly progressive glomerulonephritis, but interstitial nephritis has been observed most commonly. Treatment with Cyclosporine A or FK506 may worsen the renal pathology; however, kidney problems have been seen in patients not receiving immunosuppressive or nephrotoxic drugs.

Autoimmune hepatitis is also present in approximately 20% of patients in our cohort. Despite this, hepatosplenomegaly and lymphadenopathy caused by lymphocytic infiltrates, although prominent in Foxp3-deficient mice, are infrequent in patients with IPEX.[6,8,9] Many patients have neurologic abnormalities including developmental delay, which may be associated with being chronically ill from early childhood. Seizures also occur in some patients and may be secondary to metabolic derangements associated with severe diarrhea and diabetes.

Powell et al,[4] in their initial report, noticed increased susceptibility to infections among affected male patients and suggested that this may be a direct consequence of the genetic defect, which was unknown at the time. It is now believed that the most likely cause of increased infections among affected patients may be decreased barrier

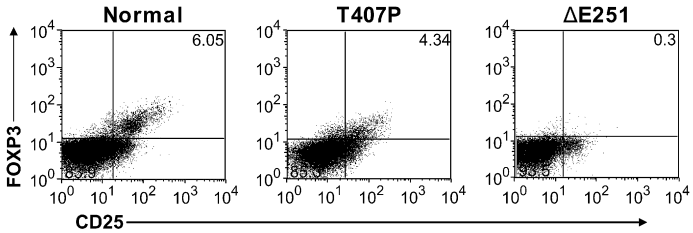

FIG 2. $CD4^+CD25^+FOXP3^+$ cells in IPEX. Flow cytometry demonstrating the population of $CD25^+FOXP3^+$ Tregs, which make up 5% to 7% of the $CD4^+$ cell population in normal individuals *(left)*. A decreased percentage of $FOXP3^+$ Treg is sometimes observed in patients with a milder IPEX phenotype and point mutations of FOXP3 *(center)*. Most patients with IPEX have a markedly decreased percentage of $FOXP3^+$ Treg *(right)*. The *heading* indicates the specific mutation present in the patient.

function of the skin and gut compounded by prolonged immunosuppressive therapy. Of the initial 50 patients in whom we identified FOXP3 mutations, more than half had serious infections including sepsis, meningitis, pneumonia, and osteomyelitis (data not shown). Sepsis caused by indwelling catheter infections was a common complication. Autoimmune neutropenia, which is present in some patients, increases susceptibility to bacterial infection. The most common pathogens observed were *Enterococcus* and *Staphylococcus* species, cytomegalovirus, and *Candida*.[3] In many cases, the infections occurred before initiation of immunosuppressive therapy.

Laboratory findings in IPEX

Most patients with IPEX have markedly elevated serum IgE, and >60% develop increased serum IgA. $CD4^+CD25^+$ T cells are present, but most patients with FOXP3 mutations have markedly decreased or absent $FOXP3^+$ Tregs (Fig 2). Otherwise, T-cell and B-cell subset quantification is normal in most patients, and T-cell proliferative responses to mitogens and antigens are typically within normal limits (data not shown).

The molecular basis of IPEX

After the discovery by Brunkow et al[11] that the characteristic phenotype of scurfy mice was the result of a mutation in the *Foxp3* gene, the human ortholog, *FOXP3,* was sequenced in several IPEX families and found to be mutated.[12-14] The human *FOXP3* gene is located on the short arm of the X chromosome and consists of 11 translated exons that encode a protein of 431 amino acids. The gene is expressed predominantly in the thymus, spleen, and lymph nodes, and particularly in $CD4^+CD25^+$ T cells, although it is inducibly expressed in activated human T cells.[15-19] FOXP3 is a member of the P subfamily of forkhead box (FOX) transcription factors, which have a highly conserved forkhead/winged-helix DNA binding domain.[20] Proteins bearing a forkhead DNA binding motif belong to a large family of related molecules that play an important role in embryonic patterning, development, and metabolism. A small subset of these transcription factors are crucial for the development and maintenance of normal immune responses (Foxj1, Foxo1, and Foxo3a), for thymic development (Foxn1), and for lineage commitment (Foxp3).[21]

FOXP3 is a transcription factor

Transcription factors are proteins that bind to specific regulatory regions within DNA and augment or suppress the transcription of particular genes. Their function is often modulated by signaling pathways that are triggered during cell development or activation. FOXP3 has long been suspected to be a transcription factor because of its homology to the forkhead DNA-binding proteins. It was initially shown to exert transcriptional repression on the promoters of key cytokine genes including IL-2 and GM-CSF.[22-24] Recent genome-wide screening approaches have suggested, however, that it may function more commonly as a transcriptional enhancer than a transcriptional repressor.[25,26]

In general, transcription factors have protein domains that allow them to interact simultaneously with DNA, with other protein cofactors, and potentially with the basal transcription machinery. In the case of FOXP3, there is a trans-repression domain required for suppression of nuclear factor of activated T cells–mediated gene transcription within the proline-rich N-terminus, a central domain that contains a C2H2 zinc finger and a leucine zipper that are involved in protein/protein interactions, and a C-terminal region that contains the forkhead DNA-binding domain and nuclear targeting sequences (Fig 3).[27] Mutations that affect any of these key functional domains may alter or abrogate the ability of the transcription factor to regulate gene expression. The majority of missense mutations identified to date affect the integrity of the forkhead domain, but amino acid deletions and substitutions have also been identified in the leucine zipper and trans-repression domain (Fig 3).

Recent studies suggest that FOXP3 physically and functionally interacts with nuclear factor of activated T cells, acute myeloid leukemia 1/Runt-related transcription factor 1 (AML1/Runx1), and possibly nuclear factor-κB, transcription factors that play key roles in the expression of multiple cytokine genes.[23,24,28] In complex with these proteins, FOXP3 acts as a transcriptional corepressor of cytokine promoters as originally demonstrated by Schubert et al.[22] As one might predict, transgenic mice that express multiple copies of the Foxp3 gene have decreased numbers of $CD4^+$ T cells in the peripheral blood, have decreased cellularity in lymph nodes and spleen, and are hyporesponsive to stimulation both *in vivo* and

in vitro.[29] This model suggests that Foxp3 functions as a rheostat of the immune system, with immune responses inversely proportional to the amount of FOXP3 protein expressed by T cells.

FOXP3 expression itself is very carefully controlled and limited, in the quiescent state, to the $CD4^+CD25^+$ $FOXP3^+$ Treg population. The exact elements that confer tissue-specific expression are not yet fully understood, but differential methylation and histone modification within the first intron of the gene are thought to play a role.[30,31] In addition, several factors have been shown to induce (for example TGF-β, IL-2, T-cell receptor [TCR] stimulation)[17,32,33] or repress (OX40)[34,35] FOXP3 expression.

FOXP3 and Treg

In recent years, FOXP3 has been linked directly to the generation of a subset of $CD4^+$ T cells expressing the IL-2 receptor α (IL-2Rα) chain CD25 and defined as natural Treg.[36] These cells are anergic, but on activation suppress the proliferation and IL-2 production of naive and memory effector T cells through a contact-dependent, cytokine-independent mechanism.[37] It was first shown in mice[38-40] and then in human beings[41] that FOXP3 plays a crucial role in the generation of Tregs. Scurfy mice, which do not express Foxp3, lack functional Tregs, which are of critical importance for the establishment and maintenance of self-tolerance and immune homeostasis.[42] Tregs play a major role in transplantation tolerance[43] and seem to be low in numbers and in expression of FOXP3 in patients who develop chronic graft-versus-host disease after bone marrow transplantation.[44] Lack of Tregs has been associated with autoimmune diseases in both human beings and mice.[45-48] Treatment strategies to increase Treg function have improved transplantation tolerance and autoimmune symptoms.[46]

Allergic diseases and asthma are caused by dysregulated, T_H2-biased immune responses in genetically susceptible individuals.[49] New insights into the function of Tregs have demonstrated that they play an important role in controlling exaggerated T_H2 response not only in animal models[50] but also in human beings. $CD4^+CD25^+$ Tregs were shown to play an important role in cow's milk protein allergy[51] as well as in the allergic responses to inhaled allergens.[49,52] It has recently been shown that the airway allergic response in sensitized mice is controlled by $CD4^+CD25^+$ Tregs in an IL-10 and TGF-β–dependent manner.[53] Experiments performed in mice have demonstrated that $CD4^+CD25^+$ Tregs are instrumental in the induction of oral tolerance[54,55] and that administration of oral antigen dramatically induces antigen specific $CD4^+CD25^+$ Treg numbers and function.[55,56]

In contrast to disorders in which there is a decrease or lack of Treg function, malignancies have been repeatedly associated with an increase in both the number and activity of Tregs. This has been associated with tumor progression,[57-61] and new therapies are being explored to reduce Treg activity with the hope of halting tumor growth.[62,63]

Foxp3 has itself been implicated as an X-linked breast cancer suppressor gene in a recent provocative report that demonstrated an increased rate of mammary carcinoma in heterozygous female mice carrying the scurfy mutation. In these tumors, the Foxp3 allele was inactivated and the human epidermal growth factor receptor 2/erythroblastic leukemia viral oncogene homolog 2 (HER2/ErbB2) oncogene was overexpressed.[64] Furthermore, the investigators observed deletions, somatic mutations, and downregulation of the FOXP3 gene in human breast carcinoma samples. Despite this, we have not observed an increased incidence of breast cancer among female carriers of FOXP3 mutations in the cohort of IPEX families that we have investigated (T. Torgerson and H. Ochs, unpublished data, September 2007).

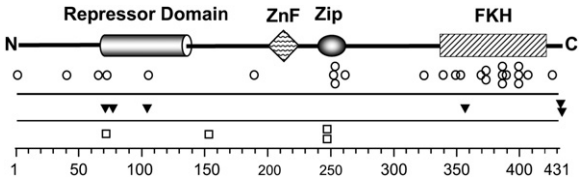

FIG 3. Domain structure of the FOXP3 protein showing the location of mutations identified in patients with IPEX. The size and location of the 4 characterized functional domains of the FOXP3 protein are shown. The *ruler* represents the amino acid number (1-431), and each *hash mark* represents 10 amino acids. The approximate locations of mutations identified in patients with IPEX are shown (O = missense point mutations, ▼= deletion/frameshift mutations, □ = splicing mutations)[7,8,10,13,14,78-81] (T. Torgerson and H. Ochs, unpublished data, September 2007). *ZnF*, Zink finger; FKH, forkhead.

IPEX and IPEX-like syndromes

To define the clinical and immunologic phenotype of IPEX and to explore a possible phenotype-genotype correlation, we have evaluated more than 100 symptomatic patients from more than 50 families for mutations in the *FOXP3* gene. In patients with a clinical phenotype compatible with IPEX, approximately 50% were found to have mutations in FOXP3. These include missense, splice site, and deletion mutations. Most missense mutations cluster in 3 specific functional domains: the proline-rich domain, the leucine zipper, and the forkhead domain (Fig 3). In one family with multiple affected members, a large deletion upstream of exon 1 was identified, resulting in expression of an abnormal protein product.[5] A point mutation affecting the first canonical polyadenylation region was found in 2 unrelated families[65] (T. Torgerson and H. Ochs, unpublished data, September 2007). By using quantitative real-time PCR, we have identified IPEX-like patients with low FOXP3 mRNA expression levels and others who have normal FOXP3 mRNA expression, suggesting that at least some IPEX-like patients have mutations involving regulatory sequences of the FOXP3 gene, such as promoter or enhancer regions. Most patients with IPEX with missense mutations and all with deletions and splice site mutations lack Tregs characterized as $CD4^+CD25^+$ $FOXP3^+$ lymphocytes (Fig 2).

Diagnosis of IPEX

A diagnosis of IPEX should be considered in any young male patient presenting with intractable diarrhea and/or

villous atrophy and failure to thrive. The presence of an erythematous/eczematoid rash or psoriasiform dermatitis, early-onset type 1 diabetes, and/or hypothyroidism strongly supports the diagnosis. Autoimmune hemolytic anemia, thrombocytopenia, or neutropenia is often present. Diagnosis of IPEX is further supported by the presence of elevated IgE and an absence of Tregs (Fig 2). The diagnosis is confirmed by mutation analysis of the *FOXP3* gene. Identification of a FOXP3 mutation allows carrier detection and prenatal diagnosis in male fetuses of known carrier females.

Treatment of IPEX

Early diagnosis is most important for the final outcome. Symptomatic treatment includes total parenteral nutrition and, if necessary, red blood cell and platelet transfusions and insulin injections. Immunosuppressive drugs have proven effective in some patients, but usually only partially and for a limited period. Cyclosporine A or FK506, with or without steroids, have been used successfully.[6,8,66] Recent data suggest that sirolimus is better tolerated and less nephrotoxic.[67] An additional advantage of sirolimus may be the fact that it allows Treg expansion while growth of effector T cells is inhibited.[68,69] A long list of other immunosuppressive medications including methotrexate, steroids, infliximab, and rituximab have been tried alone or in combination with mixed success. Furthermore, chronic immunosuppressive therapy may facilitate opportunistic infections. Stem cell transplantation is currently the only effective cure for patients with IPEX. Some patients underwent complete remission of symptoms after bone marrow transplantation.[70-72] Both complete and reduced intensity conditioning protocols have been reported as successful. In most instances, endocrinopathies, especially diabetes mellitus, will persist because of permanent damage, such as destruction of insulin-producing β cells. Generally, the prognosis for patients with IPEX is poor, and if untreated, most affected boys die at an early age.

IPEX-LIKE SYNDROMES

CD25 deficiency

Because Tregs constitutively express CD4, FOXP3, and IL-2Rα (CD25) and because IL-2Rα$^{-/-}$ mice develop splenomegaly, lymphadenopathy, inflammatory bowel disease, and autoimmune hemolytic anemia, it was not surprising to find patients with an IPEX-like syndrome who have mutations of CD25. The first reported patient presented with hepatitis, splenomegaly, lymphadenopathy, and lymphocytic infiltrates in the gut, liver, and mandible, but did not have type 1 diabetes or any other endocrinopathy. Unlike typical patients with IPEX, the affected male was the offspring of consanguinous parents and presented with moderately decreased numbers of T lymphocytes. He had decreased lymphocyte proliferation in response to mitogens, failed to reject an allogenic skin graft, and subsequently developed *Candida* infections, cytomegalovirus pneumonitis, and viral gastroenteritis. Sequence analysis revealed a homozygous 4-bp deletion within the coding region of the *IL-2Rα (CD25)* gene. Because of the severe combined immunodeficiency–like features, the patient was successfully transplanted, after cytoreduction, with bone marrow from a matched sibling.[73,74]

Ten years later, after the discovery of CD4$^+$CD25$^+$ FOXP3$^+$ Tregs, a second patient with CD25 deficiency was recognized as having a typical IPEX phenotype.[75] He presented with type 1 diabetes at the age of 6 weeks, and subsequently developed severe diarrhea, inflammatory bowel disease with villous atrophy, eczema, lymphadenopathy, hepatosplenomegaly, enlarged tonsils, hypothyroidism, autoimmune hemolytic anemia, and antibody-positive neutropenia. After activation, his CD4$^+$ lymphocytes expressed FOXP3 but lacked surface expression of CD25. This patient was found to be a compound heterozygote for a single base pair insertion in 1 allele of *CD25* and a nonsense mutation in the other allele. It is unknown whether the CD4$^+$FOXP3$^+$ Tregs of this patient are able to suppress proliferation of effector cells. Murine IL-2Rα$^{-/-}$ Tregs, however, were fully able to suppress T-cell proliferation *in vitro*.[76] IL-2Rα$^{-/-}$ mice show some similarity to Foxp3$^{-/-}$ scurfy mice. They develop massive enlargement of peripheral lymphoid organs associated with polyclonal T-cell and B-cell expansion. Older IL-2Rα$^{-/-}$ mice are susceptible to autoimmune disorders including hemolytic anemia and inflammatory bowel disease.[77]

CONCLUSION

Mutations of the transcription factor FOXP3 result in the absence or dysfunction of Tregs and lead to the IPEX phenotype. A naturally occurring mutation of Foxp3 in mice causes the scurfy phenotype, and overexpression of Foxp3 in transgenic mice is associated with severe immunosuppression. Studies of these models have provided important insights into the mechanisms of immunosuppression, autoimmunity, allergy, and tolerance and may lead to novel strategies for the treatment of autoimmune diseases, allergies, graft-versus-host disease, and cancer.

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