



Immunol Allergy Clin N Am
22 (2002) 357–368

IMMUNOLOGY
AND ALLERGY
CLINICS OF
NORTH AMERICA

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome and the scurfy mutant mouse

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A clinical syndrome characterized by polyendocrinopathy, enteropathy, and immune abnormalities first was described in 1982 as a lethal disease that occurs in male infants [1]. Subsequently, additional families were described in which affected males presented during the first few months of life with diabetes mellitus, intractable diarrhea, eczema, hemolytic anemia, thyroiditis, and serious bacterial and viral infections (Fig. 1). Linkage analysis mapped the gene for this syndrome to the X chromosome at Xp11.23-Xq13.3 [2,3].

The scurfy syndrome in mice, first observed some 50 years ago, is a lethal X-linked disease characterized by uncontrolled proliferation of CD4⁺ T lymphocytes and extensive lymphocytic infiltration of lymphoid organs, liver, and skin, resulting in greatly enlarged lymph nodes, hepatosplenomegaly, and exfoliative dermatitis. Affected mice develop severe anemia and a wasting syndrome that leads to death 16 to 25 days after birth. The scurfy gene was found to be tightly linked to murine *Gata1* [4] and *Wasp* [5] on the proximal X chromosome, in a region homologous to human Xp11.23.

The murine gene responsible for the scurfy syndrome (*Foxp3*) was identified by positional cloning and mutation analysis by sequencing [6]. The clinical similarities between scurfy syndrome and immune dysfunction, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) led to mutation analysis of the human ortholog (*FOXP3*); three laboratories have reported mutations affecting the function of this DNA-binding protein [7–10].

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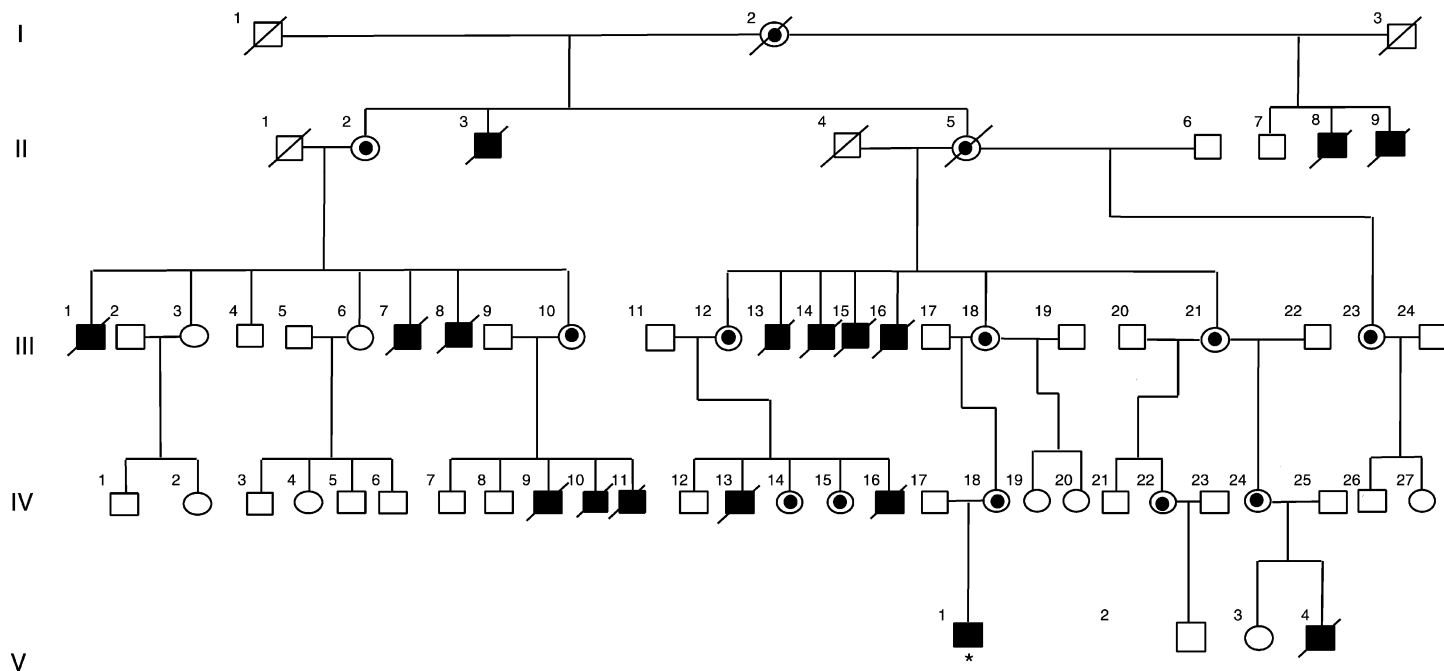


Fig. 1. Five-generation pedigree segregating a *FOXP3* mutation in a family with multiple patients with IPEX. Affected males (solid box) presented with classic findings early in life; carrier females were asymptomatic. The living affected male is marked with an asterisk. (From Bennett CL, Brunkow ME, Ramsdell F, et al. A rare polyadenylation signal mutation of the *FOXP3* gene (AAUAAA>AAUGAA) leads to the IPEX syndrome. Immunogenetics 2001;53:435–9; with permission.)

Clinical phenotype and laboratory abnormalities of IPEX

The clinical presentation, laboratory abnormalities, and strategies for treatment of IPEX have been summarized in other publications [1–3,7–21]. There is a striking clinical similarity between affected males from different families, most of whom develop symptoms shortly after birth or during the first 3 or 4 months of life. The most common findings include diabetes mellitus, severe diarrhea resulting in failure to thrive, eczema, and hemolytic anemia. Other clinical abnormalities include thrombocytopenia caused by autoantibodies, autoimmune hypothyroidism, lymphadenopathy, and splenomegaly. Most patients die during the first year of life.

There are no pathognomonic laboratory abnormalities. Serum immunoglobulin E (IgE) is elevated in most patients, and eosinophilia is common. In many instances, anti-islet cell antibodies, antienterocyte antibodies, and antithyroid antibodies have been identified; however, this finding is not consistent. Systematic evaluation of immune competence has not been performed in humans. T-lymphocyte subsets are normal, and in vitro lymphocyte proliferation to mitogens and specific antigens is present, although in some reports patients' responses were lower than that of controls. An increased response to mitogens and overexpression of interleukin-2 (IL-2) were observed in one family [22], and overproduction of multiple lymphokines by cultured peripheral blood mononuclear cells was observed in two other patients [9]. These observations, together with increased expression of MHC-II antigens [22], suggest a global activation of the immune system. Hypogammaglobulinemia, poor responses to immunization with diphtheria and tetanus toxoid, and pneumococcal polysaccharides have been reported. Assessment of immune responses has been complicated by the fact that patients often are seriously ill and malnourished and are treated with steroids, cyclosporin A, and FK506. Characteristic histopathologic findings, including severe villous atrophy and mucosal erosion with lymphocytic infiltrates within the lamina propria, have been reported within small-bowel biopsies. Lymphocytic infiltrates also have been observed in the pancreas, skin, and thyroid glands, suggesting a T cell-mediated autoimmune mechanism.

Management and treatment of IPEX

The natural course of IPEX is severe and results in death during the first or second year of life (see Fig. 1). T cell activation as indicated by MHC-II expression, increased lymphocyte production, and consistent lymphocytic infiltration of affected organs suggests a highly activated immune system. Autoantibodies that frequently are present might have a prominent role in autoimmune manifestations. Based on these observations, immunosuppressive therapy has been proposed by a number of investigators. Treatment with steroids is generally not sufficient, but the introduction of cyclosporin A and FK506 has improved significantly the long-term outcome. Despite this approach, only a few patients

have survived childhood (the oldest survivor was 11 years old in 2001) [23]. Paradoxically, some patients are immunocompromised before immunosuppressive therapy is initiated. Absent lymph node germinal centers, hypogammaglobulinemia and deficient responses to immunization have been described. Intravenous immunoglobulin has been used therapeutically when humoral abnormalities have been detected. To date, immune reconstitution has been attempted in three patients with IPEX; two received bone marrow transplants, and one received a cord blood stem cell transplant. One patient, originally reported by Wildin and investigators [10], received a bone marrow transplant from a matched sibling at 4 months of age [11]. Complete clinical and immunologic remission was achieved, and the insulin-dependent diabetes reversed to normal. The conditioning regimen, consisting of anti-T-lymphocyte globulin, busulfan, and cyclophosphamide, was effective in controlling most of the clinical features. Despite extensive conditioning, only 20% to 30% of the circulating lymphocytes were of donor origin. The chimeric state seemed stable, and the patient remained asymptomatic for more than 23 months after bone marrow transplantation; the patient then developed a fatal hemophagocytic syndrome that resulted in death 29 months after receiving the transplant. The second patient received a matched bone marrow transplant and remained asymptomatic, except for insulin-dependent diabetes, approximately 18 months following transplantation (H. Lederman, MD, personal communication, 2001). The third patient died from transplantation-related complications 3 months after a cord blood stem cell infusion (L. Whitesell, personal communication). Despite these failures, stem cell transplantation seems to be the only cure for the disease.

The scurfy mutant mouse

Because there is limited information available on the mechanisms of disease in IPEX, it is instructive to examine the murine homologue. In 1949, a spontaneous X-linked recessive mutation that leads to a fatal lymphoproliferative disease in hemizygous male mice was discovered at the Oak Ridge National Laboratory [24]. This mouse mutant was called scurfy (*sf*), because the most visible feature noted at the time was the scaliness of the skin, which resembled eczema. Further analysis revealed multiorgan lymphocyte infiltration that lead to greatly enlarged lymph nodes, hepatosplenomegaly, and exfoliative dermatitis. Lethality occurs in hemizygous *sf* males at about 3 to 4 weeks of age. Other clinical signs include severe runting, diarrhea, conjunctivitis, thickening of the ears, elevated leukocyte counts, hypergammaglobulinemia, severe anemia, and hypogonadism [25,26]. A more detailed analysis of the affected mice showed that the spleen and lymph nodes were enlarged greatly compared with those of wild-type littermates and that there was a complete loss of normal cellular architecture in these organs. Although the thymus in young (age, 14–18 days) scurfy mice were smaller compared with that of their littermates, these mice possessed distinct cortex and medullary regions [25].

Studies of adoptive transfer and antibody depletion demonstrated that the effector cells in scurfy disease are CD4⁺ T cells [25,27,28]. Whereas the adoptive

transfer of CD4⁺ T cells from hemizygous *sf* males into immunodeficient mice (such as nude mice) transfers scurfy, as characterized by the wasting and skin lesions, grafting of *sf* thymus into normal mice does not transmit the disease [28]. Transferring a mixture of *sf* CD4⁺ T cells and normal T cells into mice with severe combined immune deficiency does not transfer scurfy disease, suggesting that CD4⁺ T cells from immunocompetent mice are capable of inhibiting *sf* cells. Previous studies demonstrated that purified CD4⁺ T cells from *sf* mice are hypersensitive to stimulation through the T cell receptor (TCR). These cells, however, require two activation signals for maximal activation, one through the TCR and the other through a coreceptor such as CD28 [29]. Data have demonstrated that engagement of the TCR is required for the pathology, because *sf* males with a TCR that recognizes a single antigen (D011.10 transgene specific for albumin peptide) are not susceptible to scurfy in the absence of specific antigen [30]. The pathology observed in scurfy mice seems to result from an inability to appropriately regulate T cell function.

Flow cytometric analyses of spleens and lymph nodes from scurfy mice show a progressive increase in the percentage of Mac-1⁺ cells and a decrease in the percentage of B220⁺ cells with time. The proportion of CD4⁺ T cells also increases slightly in scurfy animals [29]. Analysis of *sf* CD4⁺ cells revealed an increased number of cells that express activation-related cell-surface markers, including CD44, CD69, CD25, CD80, and CD86 [27,29]. These cells have an increased expression of a number of cytokines, most notably granulocyte-macrophage colony-stimulating factor, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, interferon- γ , and tumor necrosis factor- α , that is consistent with the range of pathologic symptoms seen in these mice [27,29,31].

The scurfy mutation was mapped to the proximal portion of the X chromosome [25–27,32] in close linkage with the sparse-fur (*spf*) locus, which results from a mutation in the ornithine transcarbamylase gene *Otc* [33]. This region is homologous to the proximal portion of human Xp, which harbors the gene for Wiskott-Aldrich syndrome (WAS; OMIM 301000) [17]. Scurfy at one time was proposed to be the murine equivalent of WAS based on the shared features of eczema, diarrhea, and anemia [26]. The identification of the gene responsible for WAS [34] allowed mutation analysis of the murine ortholog *Wasp*; however, no changes in the expression of the gene were observed in the scurfy mouse, and no mutations were detected in the coding sequence of *Wasp* [5].

Similarly, the refinement of the IPEX locus to a 17 cM region of the X chromosome at Xp11.23-Xq13.3 [2,3] made *WASP* a reasonable candidate gene for that disorder, despite clear differences in the clinical manifestations of IPEX and WAS. No mutations of *WASP* were found in three different IPEX pedigrees examined.

Molecular basis of scurfy

Using a backcross strategy, the scurfy mutation was localized to a <300-kilobase region on the mouse X chromosome that included a region homologous

to human Xp11.23-p11.22 [6,35]. In mice, mutation analysis of all the genes in this region identified a candidate gene (*Foxp3*) that functionally could complement the scurfy defect when expressed in its wild-type form as a transgene in the mutant background [6]. The *Foxp3* mutation is a 2-base pair (bp) insertion within exon 8 that is upstream of the DNA-binding domain and results in frame shift and a product lacking the forkhead domain [6]. In parallel, analysis of the corresponding human chromosomal region led to the discovery of mutations in the human ortholog of the gene for scurfy [7–10]. The scurfy mutation is thought to result in the loss of function of the FOXP3 protein (scurfin) for the following reasons: the functional complementation in transgenic mice as described previously is consistent with the scurfy mutation being recessive; and overexpression of the scurfy mutant form as a transgene on an otherwise normal genetic background did not result in any observable phenotype [6]. Likewise, the genetics of IPEX is consistent with the recessive loss-of-function of the human FOXP3 protein.

IPEX maps to Xp11.23-q13.3 and is caused by mutations of *FOXP3*

The gene for IPEX has been mapped independently by two linkage studies of large pedigrees to chromosome Xp11.23-q13.3 [2,3]. Following the identification of the gene responsible for the murine scurfy disease [6], analysis of genes located in the corresponding human chromosomal region identified mutations within a gene encoding the DNA-binding protein FOXP3 [7–10]. The *FOXP3* gene is one of multiple genes that encode transcription factors that are distinguished by a characteristic 100-amino acid motif originally identified in *Drosophila melanogaster* as forkhead. The forkhead (or winged-helix) domain of the human FOXP3 protein is located between amino acid residues 337 and 420 at the C-terminus. The forkhead structure contains elements that are characteristic for DNA-binding proteins. A single C₂H₂ zinc-finger domain and a leucine zipper domain exist 115 and 73 amino acids, respectively, upstream from the forkhead domain. FOXP3 represents a new member of the forkhead family of transcription regulators that are involved in processes of growth and development.

Based on the clinical and laboratory findings of IPEX, FOXP3 is required for normal T cell development and function. To date, seven of eight unique *FOXP3* mutations reported in families with classic IPEX seem to affect directly the essential forkhead DNA-binding domain or total protein expression (Fig. 2). These mutations consist of three missense mutations within the forkhead domain (371 Phe → Cys; 384Ala → Thr[x2]; 397Arg → Trp), two microdeletions (2 bp and 20 bp) that remove the native stop codon, and a splice site mutation that leads to a truncated protein lacking the forkhead domain [7,9,10]. In one family, a polyadenylation signal mutation of the *FOXP3* gene (AAUAAA → AAUGAA) resulted in unstable message and classic IPEX [8]. One patient with IPEX had an inframe deletion of nucleotides 600 to 602, predicting the removal of a glutamic acid residue delE251 that was upstream of the forkhead domain [9].

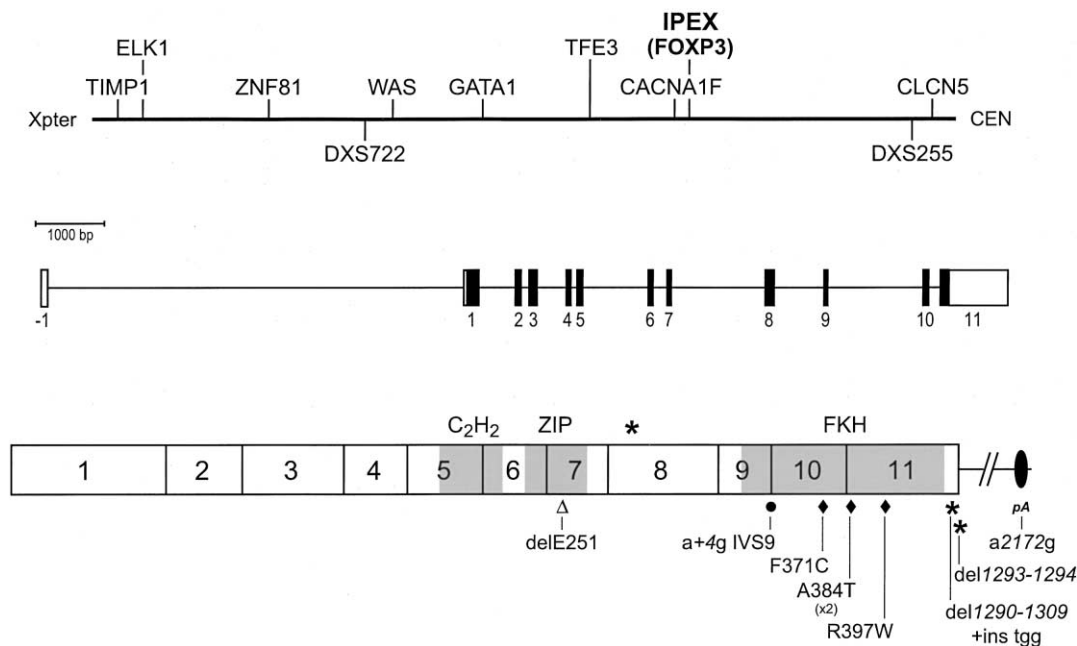


Fig. 2. Physical map of the chromosomal region surrounding the IPEX locus (*top*); exon structure of the *FOXP3* gene (*center*), with noncoding (*open box*) and coding (*solid box*) sequences; and the FOXP3 protein comprising 11 coding exons and the known functional domains, which are shaded (*bottom*). The location of 9 IPEX mutations are shown (*below the protein map*), and the location of the scurfy mouse mutation is indicated (*above the protein map* [*]). Nucleotides are indicated by lower case letters and nucleotide positions (in *italics*) of the frameshift, and polyadenylation signal (solid oval) mutations are numbered relative to the first base of the initiation codon. Single-letter codes are used for amino acid residues, and the numbering is as in the report by Brunkow and investigators [6]. The A384T missense mutation was detected in two distinct IPEX families (x2). Xpter = telomere of short arm; CEN = centromere; C₂H₂ = zinc finger; ZIP = leucine zipper motif; FKH = forkhead DNA-binding domain; open triangle (Δ) = in-frame deletion; asterisk = frameshift; ● = splicing mutation; solid diamond (◆) = missense; pA = polyadenylation signal mutation; del = deletion; ins = insertion; IVS9 = intron 9; * del = 2 microdeletions that remove the native stop codon.

Obligate carrier females were asymptomatic and were shown to have random X-chromosome inactivation [22].

Functional analysis of FOXP3

The gene for scurfy/IPEX is a novel member of a family of transcriptional regulators that is characterized by the presence of a forkhead DNA-binding domain. In keeping with the standard nomenclature devised for this rapidly growing family of proteins, the gene was designated *Foxp3* (*FOXP3* in human) [6].

The *Foxp3* and *FOXP3* genes span roughly 14.5 kilobases and are comprised of 11 coding exons and at least 1 upstream noncoding exon (see Fig. 2); in mice, there are two additional, alternatively used 5' noncoding exons. The human and mouse genes encode proteins of 431 and 429 amino acids, respectively, which are 86% similar. Expression of the gene is restricted primarily to lymphoid tissues such as the spleen and thymus [6]. More specifically, highest levels of expression are found in CD4⁺ T cells, with very little expression in CD8⁺ or B220⁺ cells. This finding is consistent with the observation that scurfy disease can be transferred to immunodeficient recipients by adoptive transfer of purified mutant CD4⁺ T cells [27].

The *Foxp3* and *FOXP3* gene product contains a region of 84 amino acids with critical sequence elements that are characteristic of a forkhead (winged-helix) domain [36–38]. This structure is comprised of three amphipathic α -helices followed by two large loops (the “wings”) and a number of conserved residues that are important for direct DNA contact. The large family of DNA-binding proteins with forkhead domains, which includes transcriptional activators and repressors, has been implicated in a wide range of biologic processes such as lineage commitment and developmental differentiation [39]. Phylogenetic analysis of all available forkhead domains from vertebrates resulted in the assignment of each domain into 1 of 17 subfamilies (see <http://www.biology.pomona.edu/fox.html>) [40]. A comparison of the FOXP3 forkhead domain with a number of other family members is shown in Fig. 3. The “P” subfamily contains an 84 residue carboxy-terminal forkhead domain in which the wing structures are considerably shorter than those found in the other subfamilies. Whether this difference also distinguishes this subfamily functionally remains to be determined. The closely related *FOXP2* gene was shown to be critical for normal speech and language development in one study in which loss of function mutations resulted in developmental verbal dyspraxia (OMIM 602081) [41].

The potential transcriptional regulatory function of FOXP3 was explored by three different experimental approaches [42]. First, confocal microscopy and cellular fractionation studies demonstrated that the FOXP3 protein is localized primarily to the nucleus and that this localization is dependent on sequences within the forkhead domain. In the second approach, electrophoretic mobility shift assays were used to show that purified recombinant FOXP3 protein and endogenous FOXP3 in nuclear extracts from stimulated T cells bind specifically

to double-stranded oligonucleotides, which include a consensus forkhead recognition sequence. In cotransfection experiments, in which FOXP3 was expressed transiently with a reporter gene whose promoter contained a consensus forkhead recognition sequence, FOXP3 repressed transcription of the reporter [42]. Whether the protein acts primarily as a transcriptional repressor in vivo, and by what mechanism, remains to be determined.

Regardless of the exact mechanism, the FOXP3 protein seems to function as an important homeostatic regulator of the immune system. When the wild-type protein is overexpressed, either in transfected cells [42] or transgenic mice [43], an effect opposite to the loss-of-function phenotype in scurfy and IPEX is observed. In the Jurkat T cell line, overexpression of wild-type FOXP3 suppresses the ability of cells to respond to activation signals, as evidenced by a dramatic decrease in IL-2 production [42]. In transgenic mice, overexpression of the wild-type allele of *Foxp3* leads to a decrease in the number and functional responsiveness of CD4⁺ T cells in otherwise normal animals. CD4⁺ T cells from these transgenic mice are hyporesponsive to stimulation in vitro and in vivo [43]. The effect of the *Foxp3* transgene seems limited to peripheral T cells, because thymic development in these mice is normal. In a separate transgenic line, high expression of a *Foxp3* transgene specifically in the thymus alters thymic development; however, this thymic expression of *Foxp3* does not seem to affect peripheral blood T cells and is unable to prevent disease in *sf* mice. These results indicate that the Foxp3 protein acts on peripheral blood T cells. The level of *Foxp3* gene expression (and presumably the encoded protein) is related directly to the overall functional competence of peripheral blood CD4⁺ T cells.

Summary

Immune dysfunction, polyendocrinopathy, enteropathy, X-linked syndrome is rare and is characterized by neonatal infantile insulin-dependent diabetes mellitus, enteropathy, endocrinopathy, eczema, and severe infections with variable immune dysfunction. Scurfy, an X-linked lethal disorder of immune regulation, is the murine equivalent of IPEX, providing a model for the human disease. The gene responsible for scurfy has been identified by positional cloning and is a member of a family of transcriptional regulators that have forkhead (winged-helix) DNA-binding domains. The cloning of the human ortholog of the mouse gene confirmed that *FOXP3* codes for the immune-specific DNA-binding protein responsible for IPEX. Missense and nonsense mutations, micro-deletions, elimination of the downstream stop codon, and mutations of the polyadenylation signal have been identified in affected members of families with classic IPEX. It has been suggested that FOXP3 acts as a transcriptional repressor in vivo. Treatment with cyclosporin A or FK506 improves the clinical course, and HLA identical bone marrow transplantation seems to provide a permanent cure.

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