



# **UNIVERSITÀ DEGLI STUDI DI ROMA "TOR VERGATA"**

**FACOLTA' DI SCIENZE MATEMATICHE FISICHE E  
NATURALI**

**DOTTORATO DI RICERCA IN IMMUNOLOGIA**

**XIX CICLO**

## **Characterization of immunologic mechanisms in rare childhood chronic inflammatory diseases: Implication for specific therapeutic intervention**

**CANDIDATA**

Dott.ssa FLAMINIA MURATORI

**RELATORE  
COORDINATORE**

Dott. FABRIZIO DE BENEDETTI  
Prof. PAOLO ROSSI

# Index

|                                                                            |    |
|----------------------------------------------------------------------------|----|
| <b>1. ABSTRACT</b>                                                         | 4  |
| <b>2. INTRODUCTION</b>                                                     |    |
| 2.1 Autoimmune and autoinflammatory disorders                              | 6  |
| 2.2 Hereditary periodic fevere syndromes                                   | 7  |
| 2.3 Febrile response                                                       | 11 |
| 2.4 The PAPA Syndrome                                                      | 12 |
| 2.5 Regulatory T cells and self tolerance                                  | 16 |
| 2.6 FOXP3                                                                  | 17 |
| 2.7 IPEX Syndrome                                                          | 19 |
| 2.8 Extracorporeal photochemotherapy                                       | 21 |
| <b>3. MATERIALS AND METHODS</b>                                            |    |
| 3.1 Mutation analysis                                                      | 23 |
| 3.2 In Vitro Cytokine Production                                           | 24 |
| 3.3 Evaluation of the Phenotype and Function of<br>Regulatory T Cells      | 25 |
| 3.4 Real-time PCR                                                          | 26 |
| 3.5 Extracorporeal photopheresis procedure                                 | 27 |
| <b>4. RESULTS</b>                                                          |    |
| 4.1 PAPA Syndrome case report                                              | 28 |
| 4.2 CD2BP1 Mutation analysis                                               | 31 |
| 4.3 Alteration of pro-inflammatory cytokine production<br>in PAPA syndrome | 33 |
| 4.4 IPEX Syndrome case reports                                             | 41 |
| 4.5 Additional information                                                 | 44 |
| 4.6 FOXP3 Mutation analysis                                                | 47 |
| 4.7 Phenotype and function of regulatory T cells                           | 49 |
| 4.8 ECP increases FOXP3 expression in CD4+ T<br>lymphocytes                | 52 |

|                                                                                    |           |
|------------------------------------------------------------------------------------|-----------|
| <b>4.9 ECP induces circulating CD4+CD25+ T cells with regulatory function.....</b> | <b>55</b> |
| <b>5. DISCUSSION</b>                                                               |           |
| <b>5.1 PAPA Syndrome.....</b>                                                      | <b>60</b> |
| <b>5.2 IPEX Syndrome.....</b>                                                      | <b>63</b> |
| <b>6. CONCLUSION.....</b>                                                          | <b>68</b> |
| <b>7. ABBREVIATIONS.....</b>                                                       | <b>69</b> |
| <b>8. REFERENCES.....</b>                                                          | <b>71</b> |
| <b>9. PUBLICATIONS AND POSTERS.....</b>                                            | <b>78</b> |

## **ABSTRACT**

The molecular defects recently identified in rare childhood immune-dysregulations outline critical steps in the pathways that contribute to the development of normal innate immune responses and the maintenance of self-tolerance. This project is based on the study of the mechanisms involved in the alteration of immune regulation, both in hereditary autoimmune diseases and in chronic inflammation, with the aim of developing a therapeutic target. Our attention has focused on:

i) A rare autosomic dominant inherited disorder of childhood named PAPA syndrome (pyogenic sterile arthritis, pyoderma gangrenosum, acne, OMIM 604410), primarily affecting the joints and skin. This disease falls under the definition of auto-inflammatory diseases, characterized by recurrent “unprovoked” inflammatory events which are not associated with high-titre of auto-antibodies or auto-antigen specific T cells. The disease has been associated with mutations in the CD2 binding protein 1 (CD2BP1). We have observed a family affected with PAPA syndrome and identified an alteration in the tumor necrosis factor-  $\alpha$  (TNF $\alpha$ ) and early interleukine-1 $\beta$  (IL-1 $\beta$ ) production. This laboratory finding was associated with a valuable therapeutic goal represented by the administration of the TNF inhibitor etanercept which resulted in a rapid, sustained clinical remission.

ii) A severe multi-organ autoimmune disease, the IPEX syndrome (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked OMIM 304790). The disease is caused by mutations in the FOXP3 gene (transcription factor fork-head box protein 3) which results in absence/alteration of the regulatory T cells (Treg) population. We have

studied two IPEX patients with a milder clinical phenotype and have identified their mutations which lay outside the FOXP3 functional DNA binding domain. The CD4<sup>+</sup>CD25<sup>+</sup> T cell population was studied and analysed. A functional impairment in the suppressive properties of these cells was found. It has been recently demonstrated that extracorporeal photopheresis (ECP) treatment in humans increases the levels of circulating regulatory T cells in vivo [1, 2]. Although the efficacy of this treatment awaits definitive proof in controlled clinical trials, data in several published series suggest that ECP represents an alternative to standard immune-suppression. Based on these findings, with a therapeutic purpose, we tempted to modulate regulatory T cells in vivo in one of the IPEX patient. Our results show that treatment with ECP leads to a significant increase in CD4<sup>+</sup>CD25<sup>+</sup> T cells with suppressive ability associated with a marked clinical response.

## **2. INTRODUCTION**

### **2.1 Autoimmune and autoinflammatory disorders**

Autoimmune disorders, estimated to affect 3–5% of the population, pose a significant public health problem [3]. Although various criteria have been applied to define classic autoimmunity, in general the destruction of the individual's own tissues seen in these diseases, is thought to result from proliferation of T lymphocytes and/or antibodies directed against self antigens. These antigens may be tissue-specific or ubiquitously expressed, and the resulting destructive inflammation may be localized, as in autoimmune endocrinopathies or systemic, as in lupus erythematosus. Many of these diseases are associated with particular histocompatibility locus antigens (HLA), suggesting that antigen presentation to T cells is important in the pathogenesis of disease [4]. When adaptive immune responses are defective there is frequently a breakdown of self-tolerance mechanisms as well. There are 3 distinct clinical syndromes which have been associated with multiple autoimmune disorders. Each is caused by a mutation in a single gene in which the primary immune defects interfere with tolerance induction and regulatory mechanisms. The autoimmune lymphoproliferative syndrome (ALPS), is characterized by lymphadenopathy, splenomegaly, autoimmune hemolytic anemia, thrombocytopenia, neutropenia, and other autoimmune manifestations. ALPS is caused by mutations in 1 of several genes involved in apoptosis [5]. The 2 other syndromes associated with multiple autoimmune disorders are both caused by mutations of transcriptional regulatory proteins. The autoimmune polyendocrinopathy

candidiasis ectodermal dystrophy (APECED) is caused by mutations in the autoimmune regulator (AIRE) gene [6], and the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is caused by mutations FOXP3 gene [7].

A separate but potentially related group of disorders comprises the so-called ‘auto-inflammatory’ diseases these include the hereditary periodic fever syndromes (HPFs). Like autoimmune disorders, these conditions involve chronic inflammation; however, auto-antibodies or antigen-specific T lymphocytes are not detected [8].

There has been considerable recent progress in identifying causative factors in auto-immune/auto-inflammatory diseases. However, effective treatments or preventatives are needed for most of these disorders [4]. These studies are based on the identification of the mechanisms involved in the alteration of immune regulation, with the aim of applying mechanism specific therapeutic approaches.

## **2.2 Hereditary periodic fever syndromes**

The first mention of periodic fever disease in medical literature probably goes back two centuries, to 1802 [9], when Herbeden described a disorder characterized by periodic pain in the abdomen and sometimes chest and extremities. Medical knowledge and research into the periodic fever syndromes have progressed over the past 200 years along a characteristic path. The first important step was a detailed clinical description and recognition of several clinical syndromes associated with periodic fever. This was followed by a brief but eventful period of genetic discoveries,

when between 1997 and 2002 each of the major syndromes was linked to mutations in its own gene. We have now reached the era of unraveling the pathophysiology: how do these gene mutations result in increased inflammation and fever ? At present, the unraveling of the pathophysiology of the periodic fever syndromes is yielding new insights into innate immunity and fever: the rare defects in these genetic disorders reveal the workings of the inflammatory response.

There are at least eight major clinical syndromes, which fall under the denomination of hereditary periodic fever syndromes, the most famous being the Familial Mediterranean Fever (FMF). Each member of this group of disorders has its specific characteristics clinical phenotypes (Table 1) [10, 11]. They are however united by a central clinical phenotype of recurring episodes of fever and other symptoms of systemic inflammation particularly involving the membranous synovial and serosal linings of the abdomen, chest and joints.

The fever attacks most often start during childhood, sometimes within the first few weeks of life but can make their first appearance in adolescence (as in FMF) or even late in adulthood, especially in TNF-receptor-associated periodic syndrome (TRAPS). Their duration can vary from hardly 1 day [e.g., familial cold auto-inflammatory syndrome (FCAS)] to weeks (e.g., TRAPS), and they can occur as often as every few days to once or twice a year. The symptoms that accompany the fever are also caused by increased inflammation. Symptoms caused by serositis are common, especially in FMF: these can include peritonitis, pleuritis, and pericarditis. Myalgia, arthralgia, or arthritis and erythematous skin lesions are also often observed.

The symptoms resolve spontaneously, and in between fever episodes, most patients feel well in most of the syndromes, except for example in



chronic infantile neurological cutaneous and articular syndrome (CINCA)/neonatal onset multisystemic inflammatory disease (NOMID) [11]. The HPFs are also term “auto-inflammatory syndromes” to underline the phenotype of seemingly unprovoked recurrent inflammation, without auto-antibodies or self-reactive T-cells, which distinguish them from autoimmune diseases [12, 13]. Although some fever attacks do have an obvious trigger, e.g., exposure to cold in FCAS or vaccination in HIDS, in most cases, the trigger remains elusive. The general hypothesis is that the inflammatory response in these patients is wrongly tuned and arises from undue or increased activation of pattern recognition molecules of the innate immune system, being either too sensitive to very minor stimuli or not turned off rapidly enough at the appropriate time. Although our knowledge of the triggers involved is still limited, it is reasonable to assume that exogenous, as well as endogenous molecules, may serve as ligands.

| Syndrome                                          | Abbreviation        | Clinical Findings                                                        | Inheritance Pattern | Gene                          | Protein                                |
|---------------------------------------------------|---------------------|--------------------------------------------------------------------------|---------------------|-------------------------------|----------------------------------------|
| Familial Mediterranean Fever                      | <b>FMF</b>          | Episodes 1-3 days, polyserositis, amyloidosis common                     | Autosomal recessive | <i>MEFV</i>                   | Pyrin                                  |
| Pyogenic sterile arthritis, pyoderma and acne     | <b>PAPA</b>         | Pyogenic arthritis, pyoderma gangrenosum                                 | Autosomal dominant  | <i>CD2BP1 (PSTPIP1)</i>       | CD2BP1 (PSTPIP1)                       |
| Cryopyrin-associated periodic syndrome            | <b>CAPS</b>         |                                                                          | Autosomal Dominant  | <i>CIAS1</i>                  | Cryopyrin (NALP3, PYPAF1)              |
| Familial cold autoinflammatory                    | <b>FCAS</b>         | Provoked by cold exposure, brief episodes (hours)                        |                     |                               |                                        |
| Muckle-Wells syndrome                             | <b>MWS</b>          | Hearing loss, amyloidosis common                                         |                     |                               |                                        |
| Neonatal onset multisystemic inflammatory disease | <b>NOMID/ CINCA</b> | Almost continuous, chronic aseptic meningitis, hearing loss, arthropathy |                     |                               |                                        |
| Blau syndrome                                     | <b>BS</b>           | Granulomatous inflammation                                               | Autosomal dominant  | <i>NOD2 (CARD15)</i>          | NOD2/CARD15                            |
| TNF-receptor-associated periodic syndrome         | <b>TRAPS</b>        | Long episodes (often wk) periorbital edema, migratory myalgia and rash   | Autosomal dominant  | <i>TNFRSF1A</i>               | TNF-receptor type (p55, CD120a, TNFR1) |
| Hyper-IgD syndrome                                | <b>HIDS</b>         | Lymphadenopathy, starts in 1st year, high IgD, attack after immunization | Autosomal recessive | <i>Mevalonate kinase (MK)</i> | Mevalonate kinase (MK)                 |

**Table 1.** Hereditary auto-inflammatory syndromes in brief (adapted from Simon et al. [11])

## 2.3 Febrile response

Fever is an adaptive systemic response to an inflammatory stimulus. During fever, body temperature is regulated at a higher level [14]. Pyrogens, substances that trigger fever, are historically divided into two groups [15]. The first group consists of exogenous substances such as components of the gram-negative bacterial cell wall (e.g. lipopolysaccharide, LPS) and other microbial products [16], which share certain molecular patterns (pathogen-associated molecular patterns or PAMPs). PAMPs are recognized by a set of receptors of the innate immune system known as Toll-like receptors (TLRs) [17, 18]. LPS is sensed by the innate immune system, mainly monocytes and macrophages, through TLR4 and is considered to be of major importance in the pathogenesis of Gram-negative sepsis. There are also certain intracellular proteins able to sense pathogenic components and mount an inflammatory response; these include nucleotide-binding oligomerization domain-leucine rich repeat (NOD-LRR) proteins.

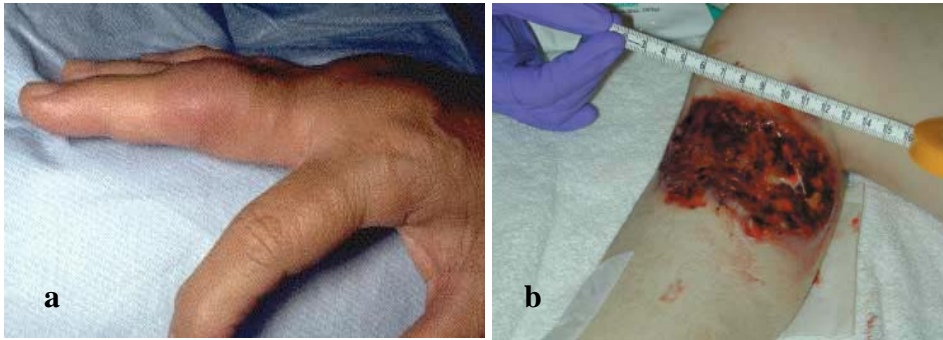
The second group of pyrogens comprises the pyrogenic cytokines, known as “endogenous pyrogens”. These include tumor necrosis factor-  $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) which are among the main mediators induced by LPS. All are able to produce a rapid onset of fever within minutes [18]. These cytokines exert their action through their own specific receptors. Fever is accompanied by a systemic reaction called the acute phase response, which is also driven by these cytokines [19]. Serum concentrations of special hepatic acute phase proteins such as C-reactive protein and serum amyloid A protein can be increased 100- to 1,000-fold, while the synthesis of a variety of other proteins is increased. The exact

function of most of the acute-phase proteins is still unclear, but there are numerous speculations about their role in the immune response. The fever episodes in the hereditary periodic fever syndromes are invariably accompanied by a marked acute-phase response. Even at times between the episodes, when the patient feels well, it is not uncommon to detect raised serum concentrations of acute phase proteins. This seems to indicate that the inflammatory cascade is turned on much more often than the patient notices and does not always reach the level of fever or other symptoms.

## **2.4 The PAPA Syndrome**

Pyogenic sterile arthritis, pyoderma and acne (PAPA) syndrome (OMIM No. 604410), a rare autosomic dominant inherited auto-inflammatory disorder of childhood, is one of the eight major auto-inflammatory syndromes currently identified [20-22]. It's clinical manifestations include early onset of recurrent and destructive inflammation of joints, skin, and muscle which can be extensive and disfiguring in some cases (Fig. 1). The joint involvement is represented by destructive recurrent arthritis affecting one to three joints at a time, and is characterized by recurring inflammatory episodes that lead to accumulation of neutrophil-rich material within the affected joints (Fig. 1a), with characteristic of septic arthritis. This ultimately results in significant synovial and cartilage destruction. Synovial fluid is purulent with neutrophil accumulation, but cultures are invariably negative. Arthritis and skin lesions have been reported to be responsive to glucocorticoids [20, 23]. Dermatologic manifestations are also episodic and recurrent, with onset usually at around 10 or 11 years of age, and are

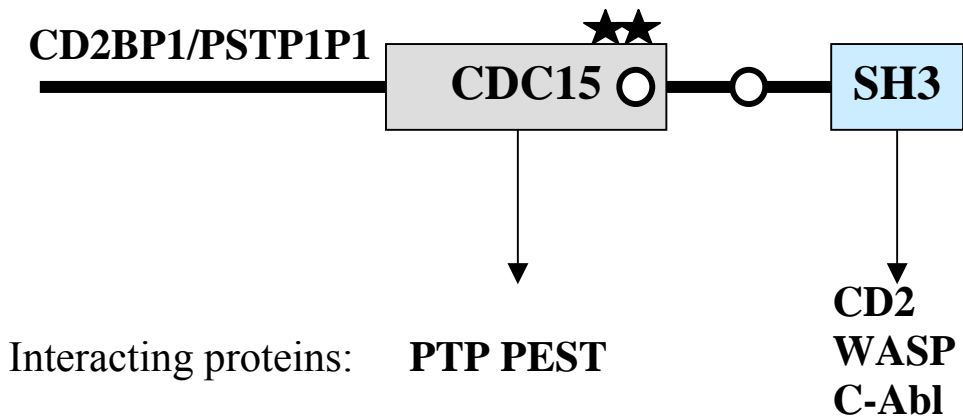
characterized by debilitating, aggressive, ulcerative skin lesions (Fig. 1b). The other component of this triad is cystic acne, which begins in adolescence and persists into adulthood



**Fig.1 (a)** Joint involvement, represented by destructive arthritis and accumulation of neutrophil-rich material within the affected. **(b)** Pyoderma gangrenosum lesion on right lower extremity. (Dorothee S Stichweh 2005)

The disease is caused by two missense mutations in the CD2 binding protein 1 (CD2BP1) gene, also known as phosphatase- proline serine threonine interacting protein 1 (PSTPIP1) [21], situated on chromosome 15q [22]. The CD2BP1 gene encodes for an adaptor protein known to act as a bridge between PEST-type protein tyrosine phosphatases (PTPs) and both the Wiskott Aldrich syndrome protein (WASP) and cAbl tyrosine kinase [24-26] (Fig.2). CD2BP1 is expressed predominantly in hematopoietic tissues and the lung [27, 28] and is the mammalian homologue of the yeast *Schizosaccharomyces pombe* CDC15p, a tyrosine-phosphorylated protein involved in the organization of the cytoskeleton [29]. CD2BP1 is found in association with cortical actin and with lamellipodia during most of the cell cycle and migrates to the cleavage furrow during cytokinesis [28]. Ectopic expression of CD2BP1 in mice induces formation of filopodial membrane

extensions, suggesting a role in actin reorganization [28]. In T lymphocytes, CD2BP1 serves as an adaptor protein bringing the cell-surface CD2 molecule into complexes with WASP, thereby coupling the T cell receptor complex with the actin cytoskeleton to promote the formation of the “immunological synapse” [24].



**Fig.2** CD2BP1 structure and protein/protein interactions. The CDC15-like and SH3 domains are shown boxed. Clear circles are PEST-rich sequences, and stars represent the E250Q and A230T mutations. Interacting proteins are shown below their associated domain

Dephosphorylation of CD2BP1 occurs through the interaction of the tryptophan at residue 232 of its coiled-coil domain with the C-terminal proline-rich homology domain of PEST-type PTPs. Both disease-causing missense mutations lie in the coiled-coil domain of CD2PB1 and almost completely abolished the interaction with the PTP PEST-type. The mutant proteins should therefore be hyper-phosphorylated. Quantitative two-hybrid

experiments revealed no detectable differences in the interaction of CD2 or WASP with mutant CD2BP1, relative to wild type CD2BP1[21].

It has also been demonstrated that CD2BP1 protein also binds, and has a similar cellular and sub-cellular distribution, to pyrin, the protein coded by the MEFV (for Mediterranean fever) gene and whose mutations cause FMF [30]. PAPA-associated CD2BP1 mutants exhibit increased binding to pyrin. [30]. This is the result of tyrosine hyper-phosphorylation. In this regard it is notable that the PAPA associated mutations A230T and E250Q, increase the interaction between CD2BP1 and pyrin, whereas the Y344F mutation (not PAPA-associated) reduces the interaction. Pyrin, by inhibiting caspase-1, acts as a inhibitory protein for the inflammasome/proIL-1 $\beta$  complex and subsequent processing of IL-1 $\beta$  [31]. The abnormal interaction of the mutated CD2BP1 with pyrin may thus indirectly modulate inflammasome activity.

To our attention came M.C., a 22 year-old boy whose diagnosis of PAPA syndrome was based on clinical data. We identified the mutation in the CD2BP1 gene. Given the bad clinical conditions of M.C. and the poor response to cortisone and cyclosporine A, we decided to study the pro-inflammatory mediators involved in PAPA syndrome with the therapeutic goal of adjusting the pharmacological treatment.

## 2.5 Regulatory T cells and self tolerance

The immune system must be capable of mounting an effective immune response against foreign/microbial agents, but must not be self-reactive. Mechanisms at both central and peripheral levels exist to maintain tolerance against self-antigens [32]. Centrally, self-reactive clones are eradicated during thymocyte differentiation, and peripherally, various mechanisms exist to control the self-reactive clones that have escaped central tolerance. Among these,  $CD4^+CD25^+$  regulatory T cells (Treg) play a critical role in the maintenance of peripheral immunological tolerance by limiting autoimmune processes and inflammatory responses [32, 33]. Neonatal thymectomy in mice and thymic hypoplasia in humans (DiGeorge syndrome) result in impaired Treg generation and in the development of organ-specific autoimmune diseases [34, 35]. Treg generation in the thymus requires interaction with MHC class II molecules expressed by cortical epithelial cells [36]. Other signals have also been implicated, including those delivered by CD28, CD40 ligand, and cytokines ( $TNF\alpha$  and  $TGF\beta$ ) [37]. However, IL-2, which is necessary for Treg survival in the periphery, is dispensable for their development [37]. Treg represent a small subset of  $CD4^+$  T cells (5–10%). They express several surface markers including CD25 (the IL-2 receptor  $\alpha$ -chain), CTL-associated antigen 4 (CTLA4), and glucocorticoid-induced TNF receptor (GITR). However, these molecules are up-regulated in naive  $CD4^+CD25^-$  T cells following TCR stimulation and cannot therefore serve as absolute Treg markers.

Treg are anergic in vitro. They fail to proliferate and secrete IL-2 in response to TCR ligation, and they depend on IL-2 generated following



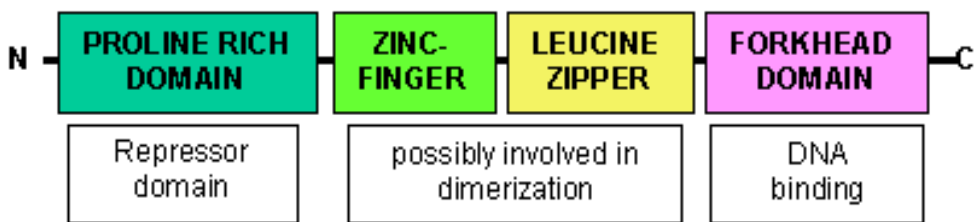
activation of CD4<sup>+</sup>CD25<sup>-</sup> T cells to survive and exert their function. The mechanisms mediating the suppression of immune responses in vivo by Treg are still unclear. Several studies support the involvement of contact-dependent inhibition, while others suggest that Treg can exert their function by secreting immunosuppressive cytokines such as IL-10 or TGFβ or by directly killing their target in a perforin-dependent manner [38]. The transcription factor fork head box protein 3 (FOXP3) has been identified as a molecular marker, and a key regulatory gene, for the development and function of Treg cells [39]. FOXP3 expression seems to be largely restricted to a small subset of T cells and defines 2 pools of Treg with suppressive activity: CD4<sup>+</sup>CD25<sup>+</sup>high cells and a minor population of CD4<sup>+</sup>CD25<sup>+</sup>low T cells. Ectopic expression of FOXP3 in vitro and in vivo is sufficient to convert naive murine CD4<sup>+</sup> T cells to Treg [40]. In contrast, over expression of FOXP3 in human CD4<sup>+</sup>CD25<sup>-</sup> T cells in vitro is not sufficient to generate potent suppressor activity, suggesting that additional factors are required for bona fide Treg activity [41]. In humans, cross-linking of the TCR and CD28 stimulation, or antigen-specific stimulation, can induce CD4<sup>+</sup>CD25<sup>-</sup> FOXP3<sup>-</sup> cells to become Treg, express FOXP3 and exert suppressor function [40]. These results suggest that de novo generation of Treg may be a natural consequence of the immune response in humans.

## **2.6 FOXP3**

Human FOXP3 is a 47 kDa protein member of the forkhead/winged-helix family of transcription factors. The members of the fox family are both transcriptional repressors and activators, and have a forkhead (FKH) domain

(Fig. 3), which is critical for DNA binding and nuclear localization [40]. FOXP3 acts as a transcriptional repressor for promoters of key cytokine genes such as IL-2 and GM-CSF [42, 43]. Recent studies suggest that FOXP3 physically and functionally interacts with transcription factors that play key roles in the expression of multiple cytokine genes such as nuclear factor of activated T cells (NFAT), acute myeloid leukaemia 1/Runt-related transcription factor 1 (AML1/Runx1), and possibly nuclear factor- $\kappa$ B (NF- $\kappa$ B) [42, 44, 45].

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, the proline-rich N-terminus is a trans-repression domain required for suppression of NFAT mediated gene transcription, a central domain that contains a C2H2 zinc finger, a leucine zipper involved in protein/protein interactions, and a C-terminal region that contains the forkhead DNA-binding domain and nuclear targeting sequences (Fig. 3) [46]. Mutations that affect any of these key functional domains may alter or abrogate the ability of the transcription factor to regulate gene expression.



**Fig. 3** Schematic representation of FOXP3 protein showing the locations and functions of the various domains within the molecule.

## 2.7 IPEX Syndrome

The IPEX syndrome was described 25 years ago by Powell et al [47] as a syndrome of enteropathy, endocrinopathy, and fatal infections affecting young male patients. The murine equivalent of IPEX is the so called *scurfy* mutant mouse with an analogous X-linked pathology [48]. 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 [47]. IPEX is characterized by multiple autoimmune and allergic features [47, 49, 50], but it is now recognized that most patients present with a basic clinical triad of enteropathy, endocrinopathy (diabetes or thyroid disease), and dermatitis.

Early onset enteropathy, with villous atrophy and infiltrating inflammatory cells, is the most common manifestation. Clinically, this results in severe watery diarrhoea that is at times mucoid or bloody. The diarrhoea may markedly worsen after an affected infant is switched from being breast-fed to regular formula, and some patients develop severe food allergies [51]. Symptoms may or may not respond to dietary manipulation and consequently, patients often require total parenteral nutrition to reverse severe failure to thrive. Immune-suppressive therapy often improves but may not resolve the gastrointestinal symptoms and in most cases this difficulty is the primary cause of death. Early onset type 1 diabetes (T1D), with auto-antibodies to islet antigens, is usually present and other autoimmune endocrinopathies are common, together with thrombocytopenia, haemolytic anaemia, and arthritis [7, 50, 51].

Asthma and eczema, with increased serum immunoglobulin (Ig)E levels, are also commonly reported [50, 52]. 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 haemolytic anaemia, autoimmune thrombocytopenia, and autoimmune neutropenia, which together affect approximately 50% of patients [7, 47, 52]. Relevant auto-antibodies can frequently be demonstrated in the serum.

In human beings, 13 FOXP3 mutations have been reported [7]. Ten disrupt the forkhead domain, 2 affect the leucine zipper domain, and 1 affects the first polyadenylation signal. The forkhead domain is required, both in humans and mice, for FOXP3 function [40]. The impact of mutations in the leucine zipper domain and in the polyadenylation site on expression and function of FOXP3 is still unknown.

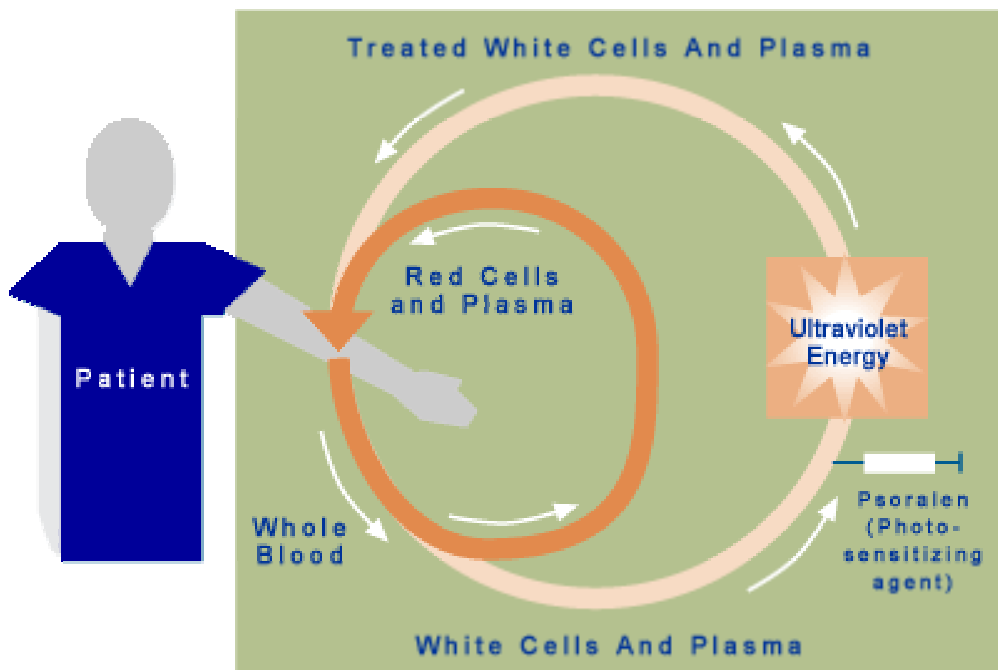
We have studied two IPEX patients with a milder clinical phenotype and have identified their mutations which lay outside the FOXP3 functional DNA binding domain. The CD4<sup>+</sup>CD25<sup>+</sup> T cell population was studied and analysed. One of the patients presented an uncontrolled enteropathy that became unresponsive to traditional immune-suppression. We therefore treated him with ECP according to the rational described in the subsequent paragraph.

## **2.8 Extracorporeal photochemotherapy**

Extracorporeal photochemotherapy (ECP) consists in exposing ex-vivo patient's peripheral blood leucocytes, obtained by a leukapheresis process, to ultraviolet-A (UV-A) irradiation in the presence of the DNA intercalating agent 8-methoxypsoralen (8-MOP) and subsequent reinfusion of autologous treated leukocytes into the patient (Fig. 4). ECP has been used in the treatment of severe refractory graft versus host disease, and to a lesser extent in the prevention of organ graft rejection [53]. Although the efficacy of this treatment awaits definitive proof in controlled clinical trials, data in several published series suggest that ECP represents an alternative to standard immune-suppression. Unlike conventional immune-suppressive therapies, patients undergoing long-term ECP have no reported risk of developing infections or acute side effects [53].

The mechanism of the immune-modulatory action of ECP remains elusive [54]. Exposure to UV-A and 8-MOP leads to apoptosis of the reinfused lymphocytes [55-57]. In animal models it has been shown that these cells localize mainly in the spleen and liver where they are scavenged by resident immature dendritic cells (DCs) which in turn develop into tolerogenic DCs [58]. It has been recently demonstrated that ECP treatment in humans increases the levels of circulating regulatory T cells in vivo [1, 2]. Based on this findings, on the poor control of clinical manifestations by conventional immune-suppression in our patient, on the observation that the mutation carried by the patient allowed the generation of some normal FOXP3 mRNA, we hypothesized that treatment with ECP in this patient could force FOXP3 expression inducing a significant increase in the amount

of normal FOXP3 mRNA leading therefore to an increase in regulatory T cells and to a better control of the clinical manifestations.



**Fig.4** Extracorporeal photopheresis consists in: a) collection of PBMC from patient by leukapheresis, b) ex-vivo exposure of PBMC to UVA in the presence of the DNA intercalating agent 8-methoxypsoralene, c) reinjection of the irradiated PBMC in the patient.

## **3. MATERIALS AND METHODS**

### **3.1 Mutation analysis**

#### **CD2BP1**

CD2BP1 exons were amplified from genomic DNA through the use of oligonucleotide primers reported by Wise et al. [21]. Screening for mutations was performed by denaturing high-performance liquid chromatography (DHPLC) with the use of Wave analysis (Transgenomic, San Jose, Calif), using conditions recommended by WaveMaker 2.0 software. Polymerase chain reaction amplicons were purified with the Nucleospin kit (Macherey-Nagel, Düren, Germany) and sequenced with CEQ DTCS (Beckman, Fullerton, Calif), with the use of a CEQ 2000 automated sequencer (Beckman).

#### **FOXP3**

The 11 exons, including the intron-exon junctions, of the FOXP3 gene were amplified from genomic DNA by polymerase chain reaction (PCR), sequenced by the Big Dye Terminator kit version 1.1 (Applied Biosystems, Warrington, Cheshire, UK) and the sequences were run on an ABI Prism 377 (Applied Biosystems). A panel of 200 control men was analyzed by DHPLC to exclude that the nucleotide variations detected in the 2 patients were polymorphisms.

For the expression analysis in patient 1, the total RNA was obtained from peripheral blood mononuclear cells (PBMC) using TRIzol (Invitrogen,

Carlsbad, CA). Reverse transcription was performed with the Advantage reverse transcription for PCR kit (Clontech, Palo Alto, CA). Complementary DNA (cDNA) spanning from exons 1 to 5 was amplified by PCR, with specific primers. Transcripts were cloned by TOPO TA Cloning kit (Invitrogen, Life Technologies Inc.) and selected colonies, representing all the PCR products observed, were sequenced.

## **3.2 In Vitro Cytokine Production**

Peripheral blood mononuclear cells (PBMC) from 15 healthy control subjects (age range, 6 to 21 years), 15 patients with oligoarticular juvenile idiopathic arthritis (oligo-JIA) (age range, 4 to 18 years), and unaffected and affected individuals of the family with PAPA syndrome were separated by means of Ficoll/Histopaque. Three samples of individual IV-2, one obtained before etanercept treatment, were available;  $2 \times 10^5$  PBMC were cultured in RPMI 1640 (GIBCO, Invitrogen Corp, Carlsbad, Calif) with 10% FBS (HyClone, Logan, Utah) at 37°C in 5%CO<sub>2</sub> in a 96-well plate with or without 10 ng/mL of lipopolysaccharide (LPS) (*Escherichia coli*, 055:B5; Sigma, St Louis, Mo) for the indicated time. A neutralizing monoclonal antibody to human interleukin (IL)-1 $\beta$  (R & D Systems) was added at 10 mg/mL. Otherwise for tolerization experiments, PBMC were pre-activated with LPS for 8 hr and re-stimulated with 10ng/ml LPS for 24 hr. The supernatants were harvested, centrifuged, and frozen at -80°C. IL-1 $\beta$ , IL-6, and TNF $\alpha$  levels were measured with commercial ELISA (R&D



Systems, Minneapolis, Minn, and Biosource Europe, Nivelles, Belgium), according to the manufacturer's instructions.

### **3.3 Evaluation of the Phenotype and Function of Regulatory T Cells**

At the time of sampling, the IPEX patient 1 was receiving prednisone (.5 mg/kg/day) and cyclosporin A (7 mg/kg/day), and the IPEX patient 2 was receiving prednisone (2 mg/kg/day) and azathioprine (2 mg/kg/day). Two independent blood samples from the 2 patients were analyzed. PBMCs from the two IPEX patients were prepared by centrifugation on Ficoll-Hystopaque. Samples from 4 patients with autoimmune diseases treated with the same immune-suppressants (Table 3) were used as disease/treatment controls. Ten healthy young adults (age, 22–40 y) served as normal controls. Monoclonal antibodies for flow cytometry and sorting were from Becton-Dickinson (San Jose, CA). For flow cytometry,  $5 \times 10^5$  PBMCs were stained with appropriate antibody combinations of fluorescein, phycoerythrin, antigen-presenting cells, and cytochrome-conjugated monoclonal antibodies; in particular, FN50 (anti-CD69), M-A251 (anti-CD25), RPA-T4 (anti-CD4), UCHT1 (anti-CD3), G42-8 (anti-CD8), HIB19 (anti-CD19), and 3G8 (anti-CD16). Cells were analyzed by FACSCalibur (Becton-Dickinson). For sorting, PBMCs were stained with fluorescein isothiocyanate–conjugated anti-CD8, CD16, and CD19 antibodies, and PE-conjugated anti-CD25 antibodies.  $CD4^+$  T cells were identified as CD8, CD16, CD19, and CD25 negative. The  $CD4^+CD25^+$  T cells, containing Treg

cells, were negative for all markers, except for CD25. The cell populations were separated using the FACS Table Vantage SE (Becton-Dickinson).

$2 \times 10^4$  cells per well of sorted T cells (total  $CD4^+$  or  $CD4^+CD25^-$ ) were cultured for 4 days in triplicate in 96-well round bottomed plates (Costar) in the presence of  $2 \times 10^4$  irradiated (3000 rad) autologous PBMCs in 200  $\mu$ l/well of serum-free X-VIVO-15 medium (BioWhittaker, Verviers, Belgium). Cells were stimulated with 2  $\mu$ g/ml of soluble anti-CD3 (clone OKT3, ATCC, Manassas, VA, USA) and 0.15  $\mu$ g/ml of soluble anti-CD28 (Becton-Dickinson). During the final 16 hours cells were pulsed with 0.5  $\mu$ Ci/well of H-thymidine and proliferation was evaluated by thymidine uptake using a liquid scintillation counter.

### **3.4 Real-time PCR**

Total RNA was extracted using TRI-reagent™ (SIGMA®, Saint Louis, MI) or RNeasy Mini Kit™ (Qiagen, Valencia, CA), according to the manufacturer's instructions. Complementary DNA was synthesized from each RNA sample using 1<sup>st</sup> Strand cDNA Synthesis Kit for RT-PCR (AMV)+ (Roche Diagnostics, Indianapolis, IN ). Real-time polymerase chain reaction (PCR) was performed on the ABI PRISM™ 7700 Sequence Detector (Applied Biosystems, Foster City, CA) platform, using Taqman universal Master Mix (Applied Biosystems). FOXP3 expression was tested using Assays on Demand reagents (Applied Biosystems, Hs00203958\_m1). All reported mRNA levels were normalized using TaqMan® Endogenous Control, GAPDH (Applied Biosystems 4319413E). Each PCR reaction was

conducted in triplicate. Relative quantification was preformed using the comparative  $C_T$  method, as described [59].

### **3.5 Extracorporeal photopheresis procedure**

ECP consisted of discontinuous leukapheresis with an extracorporeal system (UVARR apparatus Therakos, Westchester, Penn, USA). Approximately 240ml leukocyte-enriched blood was mixed with 8-methoxypsoralen (8-MOP) (final concentration: 200ng/ml) and was exposed to UVA radiation, cells irradiated with  $2 \text{ J/cm}^2$ . The UVA treated leukocytes were then re-infused into the same patients. The protocol was approved by our institution's ethics committee and the patient gave his informed consent. Ten healthy individuals provided control PBMC. ECP protocol consisted in two procedures in two consecutive days that were performed weekly for the first month and subsequently, every other week.

## **4. RESULTS**

### **4.1 PAPA Syndrome case report**

M.C., a 22-year-old man, was seen at age 2 years because of left shoulder arthritis, abscess of the left arm, and fever. Erythrocyte sedimentation rate, C-reactive protein, and white blood cell counts were elevated (Table 2). He was treated with antibiotics and showed slow improvement. His mother had severe necrotic pustules on her face and trunk during adolescence and had development of insulin-dependent diabetes mellitus (IDDM) at 42 years of age. The maternal uncle had severe joint deformities reportedly resulting from relapsing, intensively inflammatory arthritis involving several joints during childhood and had development of IDDM at 35 years. Until 10 years of age, the patient M.C. had 3 episodes of arthritis with similar clinical and laboratory features (Table 2). Synovial fluid was purulent, but cultures were negative. The synovial biopsy showed marked infiltration of neutrophils. The tuberculin test was negative. The NBT test, serum immunoglobulin levels, lymphocyte subsets, ANA, and RF were normal. He was treated with antibiotics and showed slow improvement. He had no additional episodes for 6 years. From the ages of 16 to 19 years, he had 4 similar episodes treated with non steroidal anti-inflammatory drugs and showed slow resolution (Table 2). PAPA syndrome was diagnosed on clinical data (recurrent pyogenic arthritis with negative synovial cultures) and family history. Prednisone (2 mg/kg per day) was started, resulting in rapid and complete remission. Prednisone was tapered and discontinued in 1 month. Three months later, he had arthritis of the right hip, which was treated with

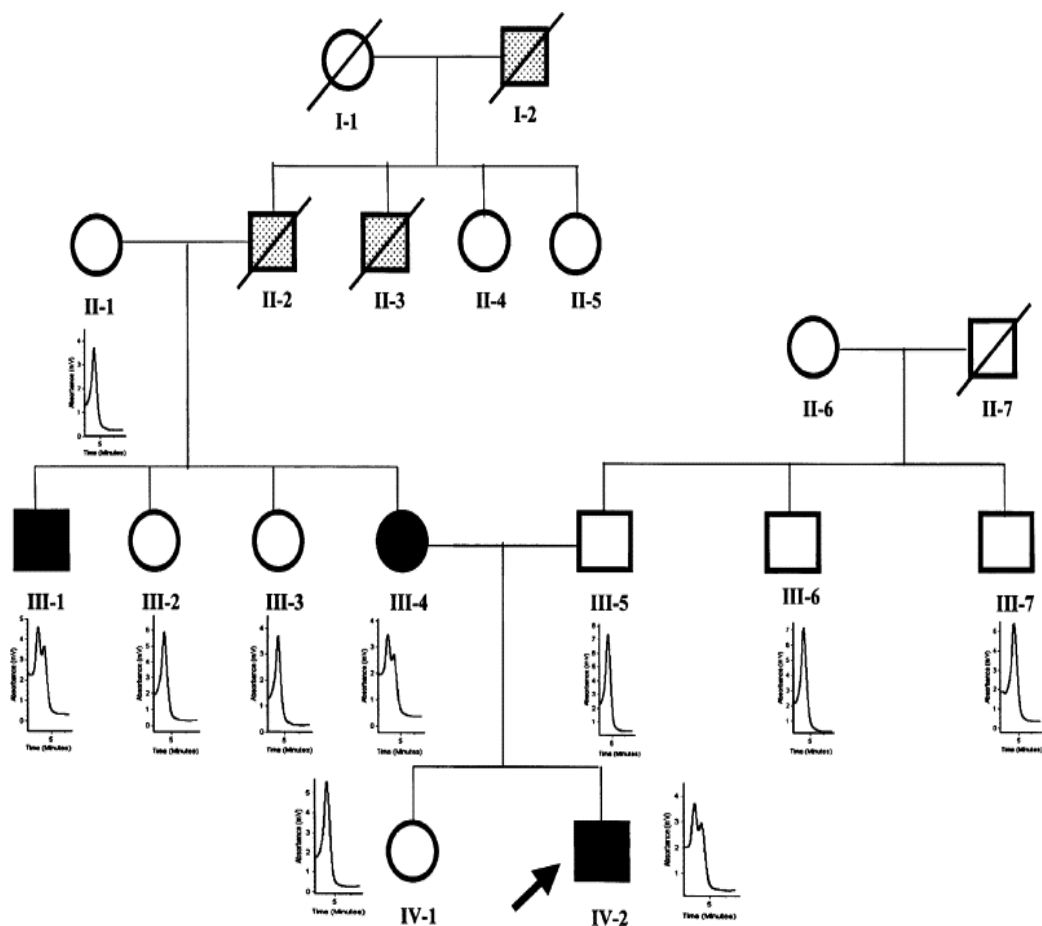
prednisone (2 mg/kg per day). Cyclosporine A (5 mg/kg per day) was added. When prednisone was reduced to 1 mg/kg per day, he had two additional episodes of arthritis of the left knee and a sterile abscess of the quadriceps muscle. Radiography showed narrowing of the joint space and a short and irregular femoral head of the right hip. He also had cystic acne and significant glucocorticoid side effects. Etanercept (25 mg/dose twice weekly), a recombinant soluble TNF receptor II/Fc fusion protein that neutralizes TNF was then started. Glucocorticoids were tapered and discontinued in 2 months. Up to now, the patient has been receiving etanercept for 30 months. No additional episodes of arthritis occurred, and cystic acne resolved. Moderate limitation of motion of the right hip persists.

| Age (y) | Affected joint        | Temperature at admission (C°) | ESR (mm/h) | CRP (mg/dL) | WBC count (10 <sup>3</sup> /mm <sup>3</sup> ) | Neutrophils (%) | SF leukocyte (x 103/mm <sup>3</sup> ) | Neutrophils (%) |
|---------|-----------------------|-------------------------------|------------|-------------|-----------------------------------------------|-----------------|---------------------------------------|-----------------|
| 2       | Left shoulder         | 39,0                          | 120        | 18,3        | 28,000                                        | 92              | ND                                    | ND              |
| 7       | Left ankle            | 39,2                          | 80         | 18,6        | 11,750                                        | 74              | 80                                    | 92              |
| 8       | Right hip             | 37,8                          | 130        | 21,3        | 12,900                                        | 81              | ND                                    | ND              |
| 10      | MCP joints (2°,3°,4°) | 39,5                          | 80         | 2,9         | 8900                                          | 61              | ND                                    | ND              |
| 16      | Right wrist           | 36,8                          | 14         | 1,5         | 8890                                          | 65              | ND                                    | ND              |
| 17      | Right ankle           | 37,8                          | 80         | 2,9         | 7300                                          | 60              | 117                                   | 96              |
| 18      | Right ankle           | 39                            | 50         | 5,8         | 11,300                                        | 68              | ND                                    | ND              |
| 19      | Right hip             | 36,8                          | 65         | 3,8         | 4000                                          | 71              | 97                                    | 87              |
| 20      | Right hip             | 37                            | 125        | 7,8         | 9670                                          | 88              | ND                                    | ND              |
| 20      | Left Knee             | 36,8                          | 54         | 2,6         | 9330                                          | 72              | 81                                    | 90              |
| 20      | Left knee             | 37,6                          | 70         | 5,2         | 10,200                                        | 74              | ND                                    | ND              |

**Table 2.** Clinical presentation and laboratory investigations of arthritis episodes in the proband individual IV-2. *ESR*, Erythrocyte sedimentation rate; *CRP*, C-reactive protein; *WBC*, white blood cell; *ND*, not determined

## **4.2 CD2BP1 Mutation analysis**

Denaturing high-performance liquid chromatography analysis of exon 10 amplimers showed heterozygosity in the proband and in two affected family members (Fig.4). Sequencing identified a wild-type and a mutant sequence with a G/A transition at position 904 in exon 10 of CD2BP1, identical to one of the reported disease-causing mutations [21]. DHPLC analysis did not reveal additional heterozygous sequence variants in both affected and non affected family members.



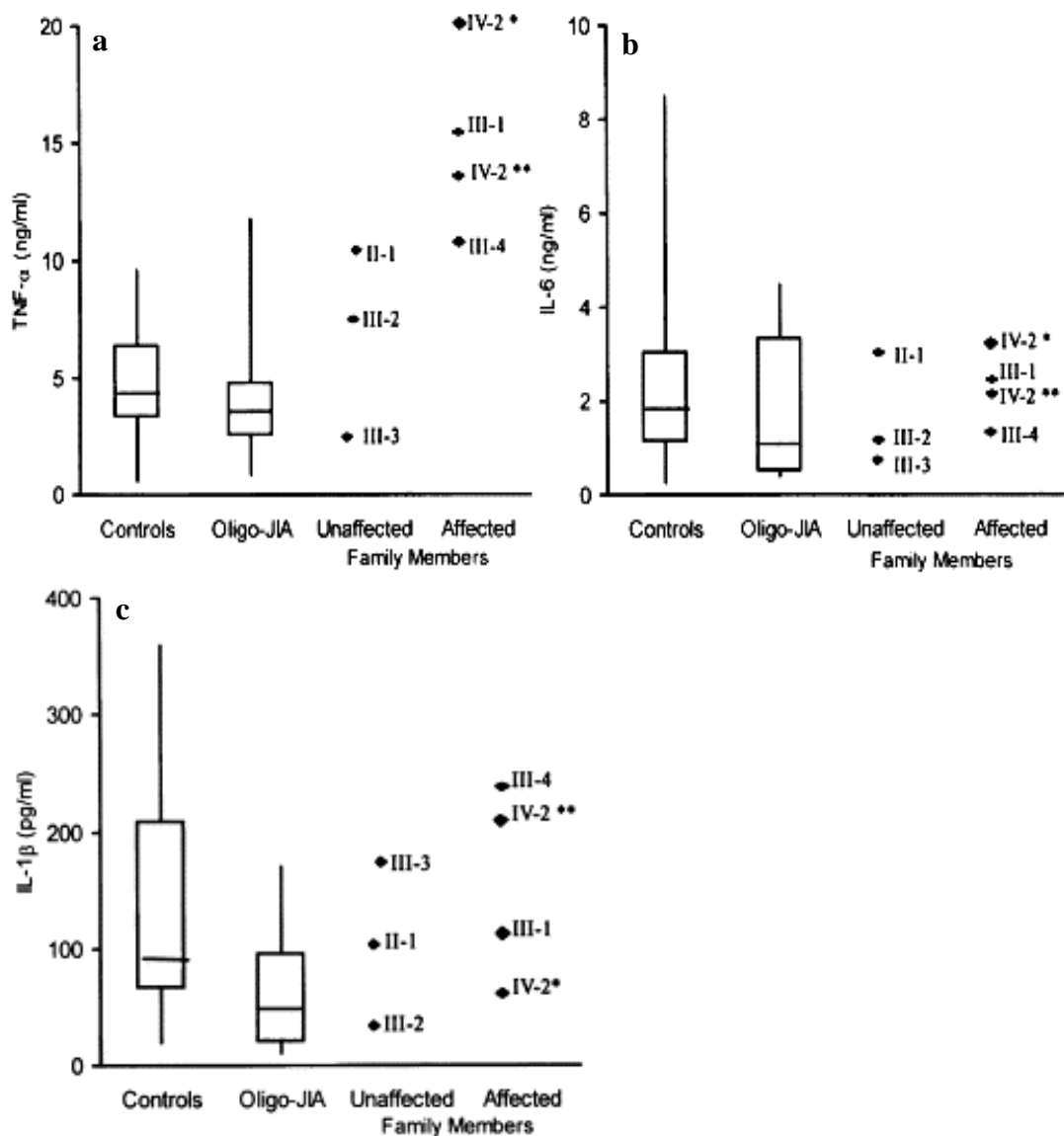
**Fig. 4** Pedigree of the family with PAPA syndrome. DHPLC profiles of exon 10 of CD2BP1 gene are shown for individuals in whom analysis was performed. Arrow, proband; line through symbol, deceased; closed symbols, affected (III-1: recurrent arthritis from age 7 years and IDDM at age 35 years; III-4: pyoderma gangrenosum at age 12 and IDDM at age 42 years; IV-2: see Case Report); open symbols, unaffected; stippled symbols, history suggestive of PAPA syndrome but unable to confirm (I-2: severely crippled by arthritis at young age; II-2: some episodes of mild, self-remitting arthritis, died at age 32 years in an accident; II-3: recurrent arthritis and diabetes at age 40 years, died at age 45 years).



### **4.3 Alteration of pro-inflammatory cytokine production in PAPA syndrome**

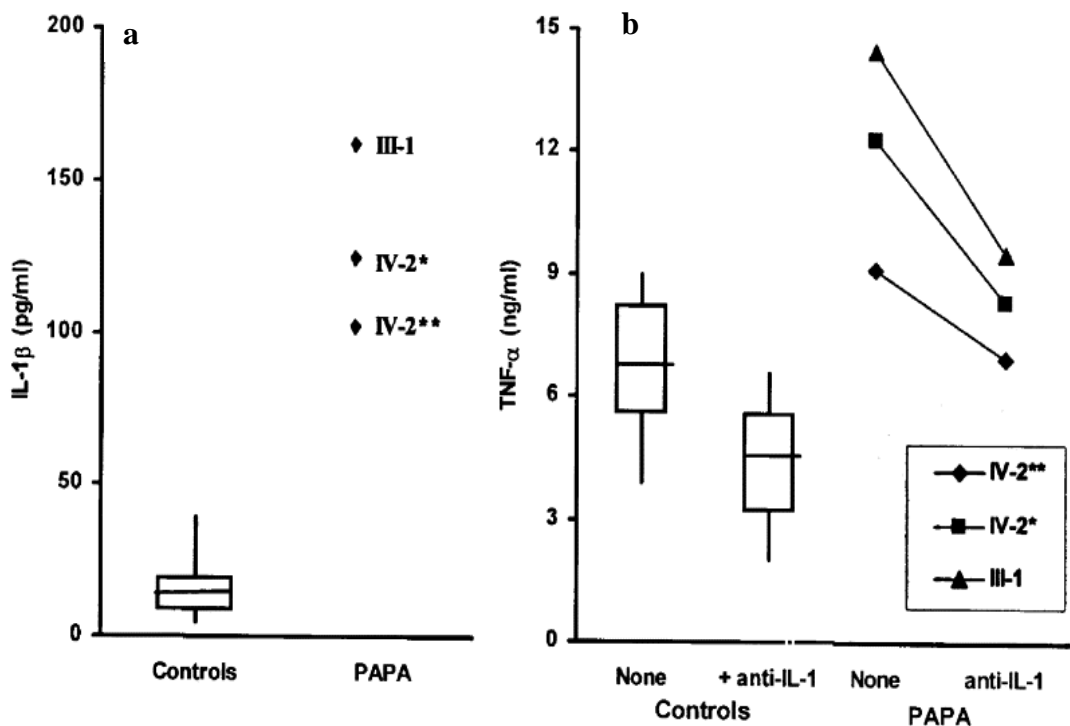
It has been hypothesized that the increase in pyrin binding seen with the PAPA mutations may alter inflammatory susceptibility by altering the balance between proinflammatory and compensatory anti-inflammatory mechanisms. LPS, sensed by the innate immune system through TLR4, is a well known stimulus for inflammatory responses. The effects of LPS are mediated by multiple signalling cascades. We studied the production of pro-inflammatory cytokines after short, long and repeated exposure to LPS.

TNF $\alpha$  production after an 18-hour LPS stimulation was higher in affected subjects than in healthy control subjects or in patients with oligoarticular juvenile idiopathic arthritis (oligo-JIA). TNF $\alpha$  production of genetically normal relatives did not differ from that of control subjects (Figure 5a). In the proband (IV-2), TNF $\alpha$  production was high before and during etanercept treatment. Individuals III-1 and III-4 and patients with JIA were not receiving glucocorticoids or TNF inhibitors at sampling. No difference was found in IL-6 and IL-1 $\beta$  production after an 18-hour stimulation (Figure 5, b and c).



**Fig. 5** Production of proinflammatory cytokines TNF $\alpha$  (a), IL-6 (b), and IL-1 $\beta$  (c) in healthy controls, in patients with Oligo-JIA, and in unaffected and affected individuals of the family with PAPA syndrome. PBMC cells were cultured in the presence of 10 ng/mL LPS for 18 hours. For healthy control subjects and patients with Oligo-JIA, results are shown as whisker box, where limits of boxes represent 25th and the 75th percentile values; lines across boxes are median values and whiskers depict minimum and maximum values. For unaffected and affected family members, results are shown as individual symbols and are the means of two independent experiments. Two samples of PBMC from individual IV-2 were tested, one obtained before (\*) and the other after (\*\*) etanercept treatment.

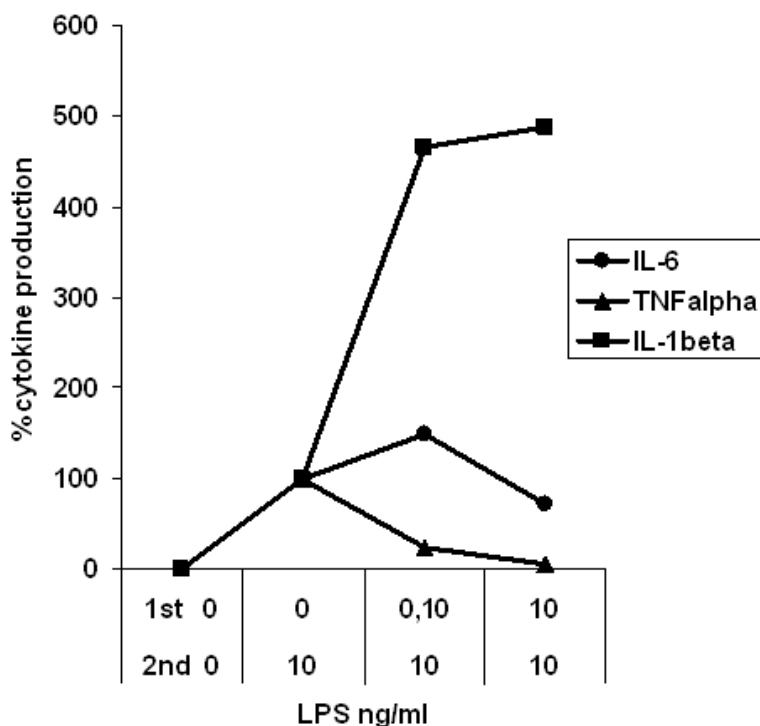
In agreement with a recent observation [30], IL-1 $\beta$  release was markedly increased in subjects with PAPA syndrome after a short-term (2-hour) stimulation (Fig. 6a). To assess whether the early increase in IL-1 $\beta$  production could be responsible for the late increase in TNF $\alpha$  production, we evaluated TNF $\alpha$  production after an 18-hour stimulation with LPS in the presence of a neutralizing antibody to IL-1 $\beta$ . Neutralization of IL-1 $\beta$  resulted in reduction of TNF $\alpha$  production in control subjects and in patients. However, in the patients, TNF $\alpha$  production was still markedly higher than that of control subjects (Fig. 6b), suggesting that the increased production of TNF $\alpha$  after a long incubation (18hours) was partially secondary to the early IL-1 $\beta$  release.



**Fig. 6** Release of IL-1 $\beta$  after short-term stimulation (2 hours) with 10 ng/mL LPS (a) and production of TNF $\alpha$  after long-term stimulation (18 hours) in the presence or absence of a neutralizing antibody to human IL-1 $\beta$  (b) in healthy control subjects and in individuals with PAPA syndrome. For healthy control subjects, results are shown as whisker box, where limits of boxes represent 25<sup>th</sup> and the 75<sup>th</sup> percentile values; lines across boxes are median values and whiskers depict minimum and the maximum values. For individuals with PAPA syndrome, results are shown as individual symbols and are the means of two independent experiments. Two samples of mononuclear cells of individual IV-2 were tested, one obtained before (\*) and the other after (\*\*) etanercept treatment.

Chronic repeated exposure to endotoxin or LPS causes a transient increase in the threshold to endotoxin challenge. This is known as “endotoxin tolerance” and represents a negative feedback mechanism to protect from endotoxin shock. While  $\text{TNF}\alpha$  is a phenotypic marker for LPS tolerance, production of other cytokines in tolerance is less clear.

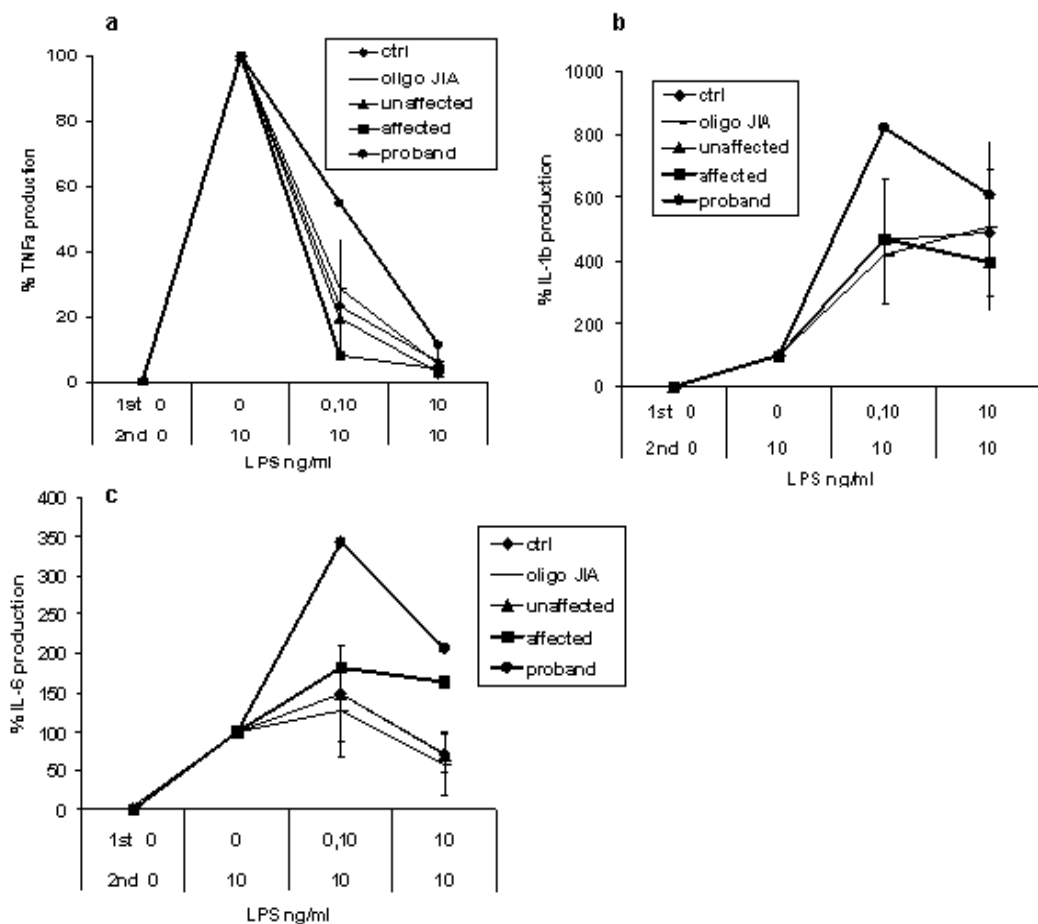
Our results show that  $\text{IL-1}\beta$ ,  $\text{IL-6}$  and  $\text{TNF}\alpha$  production, in response to LPS repeated exposure, is differently regulated (Fig. 7). PBMC were pre-stimulated with different doses of LPS for 9 hours and then re-stimulated with 10ng/ml LPS. Cytokine production was observed after this second stimulation. At each dose of LPS, cytokine level was compared to the cytokine levels of PBMC that received only the second LPS stimulation. As shown by previous studies [60-62],  $\text{TNF}\alpha$  levels showed a very strong reduction in a dose dependent manner, reaching 94% inhibition with the higher LPS dose pre-treatment.  $\text{IL-6}$  showed a different regulation pattern since low doses of the 1<sup>st</sup> LPS stimulation augmented  $\text{IL-6}$  levels, a phenomenon known as priming, while higher doses induced a modest (30%) reduction.  $\text{IL-1}\beta$  levels in contrast did not undergo any tolerogenic reduction but instead increased in a dose dependant manner.



**Fig 7.** Different cytokines respond in a different manner to LPS repeated exposure. PBMC from normal controls were pre-treated with different doses of LPS (1<sup>st</sup> LPS). After 9 hours incubation cells were washed and restimulated with 10ng/ml of LPS (2<sup>nd</sup> LPS). After 16 hr TNF $\alpha$ , IL-1 $\beta$  and IL-6 levels were determined by ELISA. The concentrations of cytokine in each sample were normalized to the concentration of the sample that received the second stimulation alone. Values are represented as percentage of the cytokine levels in samples receiving the second stimulation alone. (●) IL-6, (▲) TNF $\alpha$ , (■) IL-1 $\beta$

When we studied PBMC from the PAPA affected patient and his affected relative, a perturbation in endotoxin tolerance was observed only for IL-6. PBMC pattern production for  $\text{TNF}\alpha$  and IL- $1\beta$  in response to LPS pre-treatment reflected that observed for normal controls, oligo-JIA and unaffected family member (Fig 8a, 8b). In contrast IL-6 production was altered. No tolerance was observed for the higher LPS dose pre-treatment (Fig 8c). The proband also showed a very high priming, the percent increase of IL-6 levels for the lower dose of LPS was twice that of normal controls or oligo-JIA.

Although our patient showed a complete therapeutic response to TNF inhibition, these data suggest that mutation in CD2BP1 leads to a complex perturbation of the regulation of inflammatory cytokine production. The possibility exists that other cytokine inhibitor (i.e. anti-IL-6 receptor) may be of therapeutic benefit in these patients



**Fig 8.** PBMC were treated as already described with different doses of LPS (1<sup>st</sup> LPS). After 9 hours incubation cells were washed and restimulated with 10ng/ml of LPS (2<sup>nd</sup> LPS). Cytokines levels were determined by elisa after 16 hr incubation. a) TNFα b) IL-1β and c) IL-6. The concentrations of cytokine in each sample were normalized as already described. (◆) controls, (—)oligo-JIA, (▲)unaffected family members, (■) affected family member, (●) proband.



## 4.4 IPEX Syndrome case reports

### Patient 1

A 22-year-old man was referred because of recurrent diffuse joint pain associated with the presence of antinuclear antibodies (ANAs). The mother died at 42 years of age of intestinal lymphoma. The patient had 3 brothers. One brother died at 1 month of age from intractable diarrhoea; diabetes was not reported. The other 2 brothers, both asymptomatic, had biopsy examination proven celiac disease and responded to a gluten-free diet. One brother died at 14 years of age in an accident; the other brother, aged 26 years, was lost to follow-up evaluation. At 18 days patient 1 developed diarrhoea and severe malnutrition. Parenteral nutrition and a subsequent diet with a peptide formula reportedly were associated with improvement and he was maintained on a gluten-free diet. At 5 years, after reintroduction of a full gluten containing diet, abdominal pain, diarrhoea, and failure to thrive relapsed. An intestinal biopsy examination showed subtotal villous atrophy and mononuclear cell infiltrate in the lamina propria (Fig.9a). Immunohistochemistry subsequently was performed and revealed an intraepithelial CD3<sup>+</sup>CD8<sup>+</sup> cell count of 42/100 epithelial cells (upper limit of normal, 40) (Fig.9b). High titers of antigliadin IgG and antiendomysial antibodies were found. Celiac disease was diagnosed. After an initial clinical improvement with a gluten-free diet, diarrhoea rapidly relapsed and failure to thrive persisted. Two years later a second intestinal biopsy examination showed similar findings (not shown). IgE levels were high (200 mU/mL; normal values for age, <60 mU/mL), reportedly in the absence of allergy. Diarrhoea and failure to thrive persisted. At 15 years, the patient

developed arthralgia and transient arthritis. Antienterocyte antibodies were detected for the first time (immunofluorescence on human duodenum; Dr. E. Bosi, San Raffaele Research Institute, Milan, Italy). A diagnosis of autoimmune enteropathy was made and treatment with glucocorticoids and azathioprine was started with partial improvement. At 18 years the patient developed well-defined, scaly, erythematous plaques on the trunk and limbs. A skin biopsy examination showed parakeratosis, Poitrier-like epidermal microabscesses, and mononuclear cell infiltrate in perivascular aggregates, consistent with psoriasiform dermatitis (Fig.9e). Chronic enteropathy remained active until admission to our unit despite continuous treatment with glucocorticoids, immune-suppressants (cyclosporin A, tacrolimus), and infliximab, alone or in combination, and intermittent parenteral nutrition. At admission, the patient was receiving prednisone, cyclosporin A, and nightly parenteral nutrition. His height was 164 cm (25th–50th percentile), and his weight was 48 kg (10th– 25th percentile). He was in fair general condition, presenting with weakness and mild signs of dehydration. Maculopapular lesions were present over the limbs and trunk. The liver was mildly enlarged. The white blood cell count was  $10,570/\text{mm}^3$ , hemoglobin level was 12.1 g/dL, platelet count was  $219,000/\text{mm}^3$ , ESR was 28 mm/h (normal, <20 mm/h), and the C-reactive protein level was 39 mg/L (normal, <5 mg/L). Metabolic acidosis (pH 7.30; bicarbonate, 13.3 mEq/L) and renal failure (serum creatinine level, 2.59 mg/dL; urea nitrogen level, 42 mg/dL) were present. Blood electrolyte, glucose, and liver enzyme levels were normal. ANAs were positive (1:160; homogeneous pattern). Antitransglutaminase antibodies were negative. The patient was treated with 3 intravenous methylprednisolone pulses (1 g/day) and parenteral rehydration. He was discharged 1 week later in good general condition with

prednisone (1 mg/kg/day in 1 daily dose), tacrolimus (.04 mg/kg/day), azathioprine(2 mg/kg/day), and nightly parenteral nutrition. His weight was 50 kg, creatinine level was 1.45 mg/dL, and bicarbonate level was 22.0 mEq/L.

## **Patient 2**

A 5 year-old boy was referred to our unit because of pain in the wrist and the left proximal interphalangeal joint of the second finger and diffuse arthralgia. His 12-year-old brother was healthy. Severe chronic diarrhoea had appeared at 14 months of age. An intestinal biopsy examination showed dysmorphic villi with no atrophy and a marked mononuclear cell infiltrate in the lamina propria (Fig.9c) and an intraepithelial CD3<sup>+</sup>CD8<sup>+</sup> cell count of 60/100 epithelial cells (upper limit of normal, 40) (Fig.9d). Antigliadin and antiendomysial antibodies were negative. The patient did not improve on a gluten-free diet. IgE levels were 74 mU/mL (normal value for age, <10 mU/mL). Antienterocyte antibodies were negative. ANAs were present (1:5120, homogenous pattern). The patient was diagnosed with an unspecified autoimmune enteropathy and treatment with prednisone, azathioprine, and exclusive intravenous nutrition was started. Substantial clinical improvement and weight gain ensued. At 18 months of age the patient developed pneumonia with pleuritis, and pericarditis a month later. He was treated with prednisone and azathioprine; treatment was tapered and eventually withdrawn at 3 years and 5 months. Since then he had been growing satisfactorily on a full gluten-containing diet. At admission, he was in good clinical condition. His height was 110 cm (50th–75th percentile) and his weight was 16 kg (10th–25th percentile). Physical examination

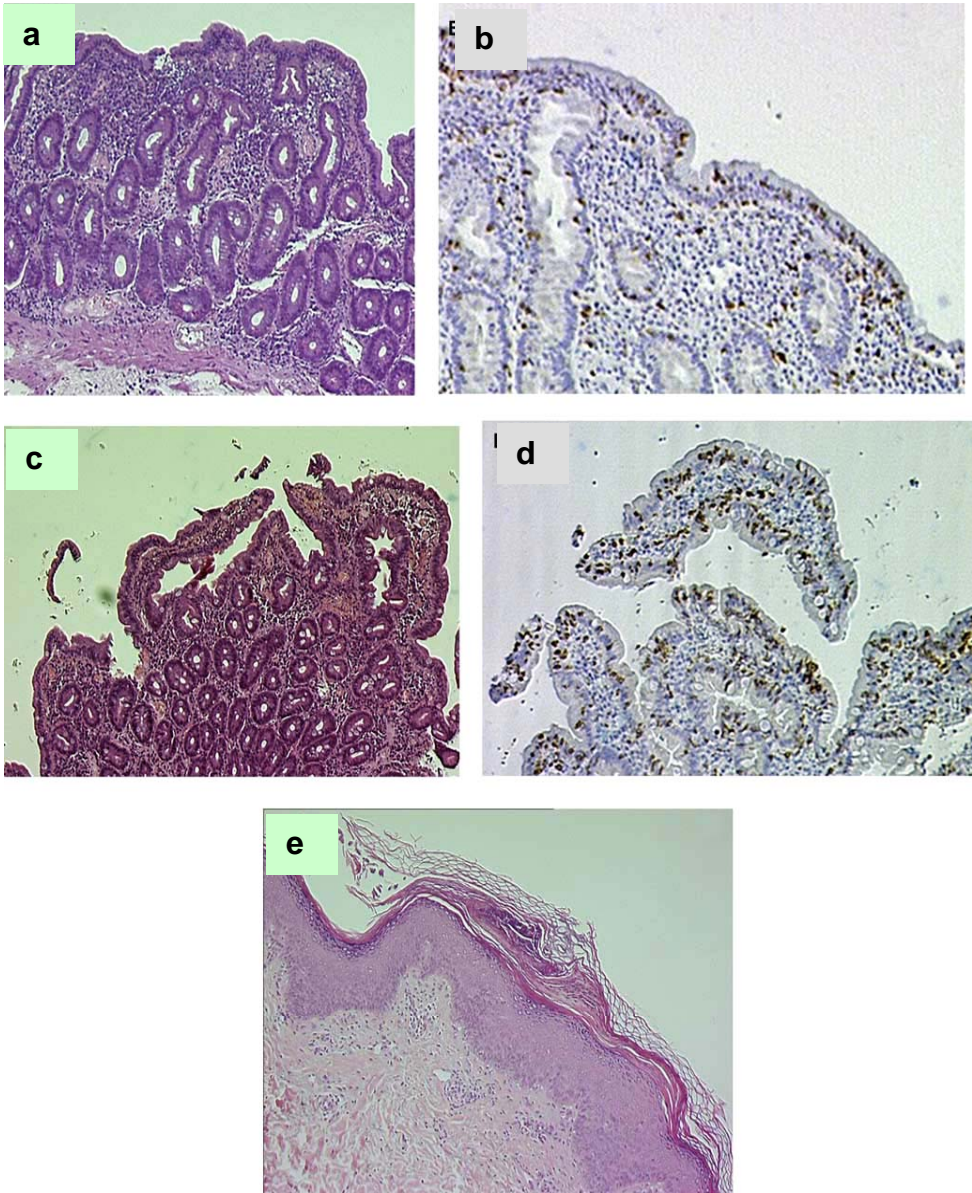
showed moderate swelling of the wrists and of the left proximal interphalangeal joint of the second finger. No other signs or symptoms were present. Blood cell counts and liver and kidney functions were normal. ESR was 34 mm/h, the C-reactive protein level was 3.3 mg/L. ANAs were confirmed at a titer of 1:5120 with a homogenous pattern. He was treated with naproxen (15 mg/kg/day). In the subsequent weeks, arthritis and arthralgia persisted and he presented with occasionally watery diarrhoea. Treatment with prednisone and azathioprine was started and was followed by a rapid resolution of arthritis and diarrhoea. After 2 years, the patient is presently a 7-year-old boy in good health, regularly attending school, who has not presented with recurrences of his enteropathy, and is being treated with azathioprine (2 mg/kg/day) and prednisone (presently .2 mg/kg/day on an alternate-day regimen). ANAs still are present (1:2560). The IgE level is 7.6 IU/mL (normal values for age, <100 IU/mL).

## **4.5 Additional information**

The following investigations were normal or negative in both patients: oral glucose tolerance test, HbA1c, fructosamine, insulin-like growth factor, DHEA-S, thyroid functions, including a thyroid ultrasound, antibodies to glutamic acid decarboxylase, thyroid (thyroglobulin and thyroid-peroxidase), smooth muscle, liver-kidney microsomes, mitochondria, extractable nuclear antigens, cardiolipin, and neutrophil cytoplasm.

In patient 1 the intestinal biopsy examination showed subtotal villous atrophy, an intraepithelial lymphocyte count greater than 40%, and mononuclear cell infiltrate. This was classified as stage 3a, according to the

modified Marsh classification [63], and the persistently high titers of antigliadin and antiendomysial antibodies were consistent with a diagnosis of celiac disease. However, patient 1 did not respond to a gluten-free diet. Whether he is affected with both IPEX and celiac disease, or whether the features compatible with celiac disease are manifestations of the immune dysregulation of IPEX, is difficult to ascertain. From a practical point of view, this case indicates that in patients with early onset enteropathy and celiac disease-related antibodies, a failure to respond to a gluten-free diet should prompt the suspicion of IPEX syndrome, even in the presence of histological findings highly suggestive of celiac disease.



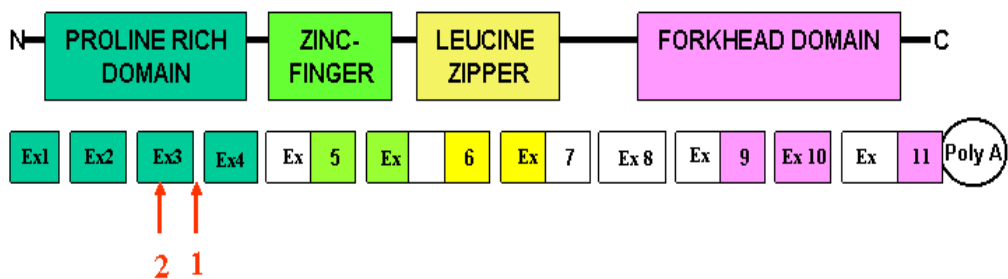
**Fig.9** Intestinal biopsy specimen of (a, b) patient 1 and (c, d) patient 2, and a skin biopsy specimen of (e) patient 1. (b, d) Immunohistochemistry of the intestinal biopsy specimen with an anti-CD3 antibody.

## 4.6 FOXP3 Mutation analysis

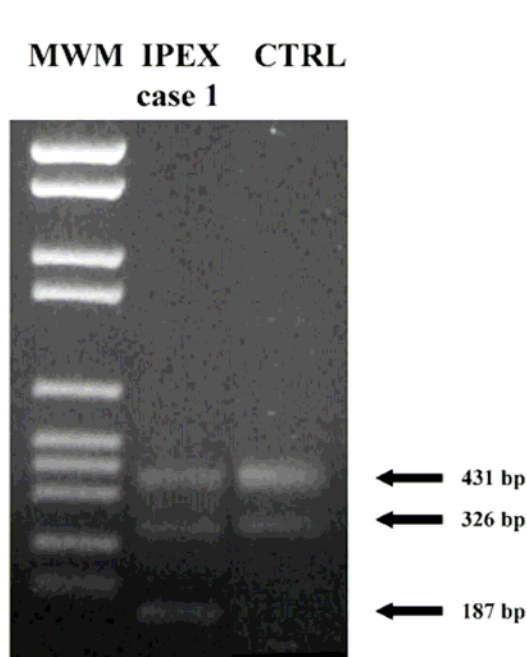
**Patient 1** carries an A>G transition at +4 in intron 3 (454 + 4 A>G), very close to the splice site (Fig. 10) . Reverse-transcription PCR was performed on RNA from PBMCs to obtain cDNA corresponding to exons 1–5. Three cDNA transcripts of 431, 326, and 187 base pairs (bp) were detected (Fig. 11). Cloning and sequencing of the 3 fragments showed that the 431-bp fragment corresponds to the full-length cDNA produced by healthy subjects; the 326-bp transcript also is found in some normal controls and represents an alternative splicing with skipping of exon 2 and conserved frame (GenBank AJ005891); in the 187-bp transcript skipping of exons 2 and 3 was seen, resulting in a frame shift. The putative resulting protein has an abnormal amino-acid composition and it is truncated owing to a premature stop codon in exon 7.

**Patient 2** is hemizygous for a C>T transition in exon 3 at nt 323 (Fig. 10), causing the substitution of a threonine with methionine at position 108 (T108M). This missense mutation affects the proline-rich domain and the resulting protein has a predicted normal forkhead domain.

The mutations of the 2 patients were not found in the screening of the 200 healthy adult men.



**Fig. 10** Schematic representation of Fcpx3 protein showing the locations of the mutations identified in IPEX patient 1 (1: A>G np+4 intron 3) and IPEX patient 2 (2: C323T (T108M)).



**Fig. 11** Fcpx3 transcripts in patient 1 and in 1 healthy control. The 431-bp fragment corresponds to the full-length cDNA produced by healthy patients; the 326-bp transcript found in the patient and in some of the normal controls represents an alternative splicing with skipping of exon 2 and conserved frame. The 187-bp transcript skips exons 2 and 3 and results in an altered reading frame with a premature stop.

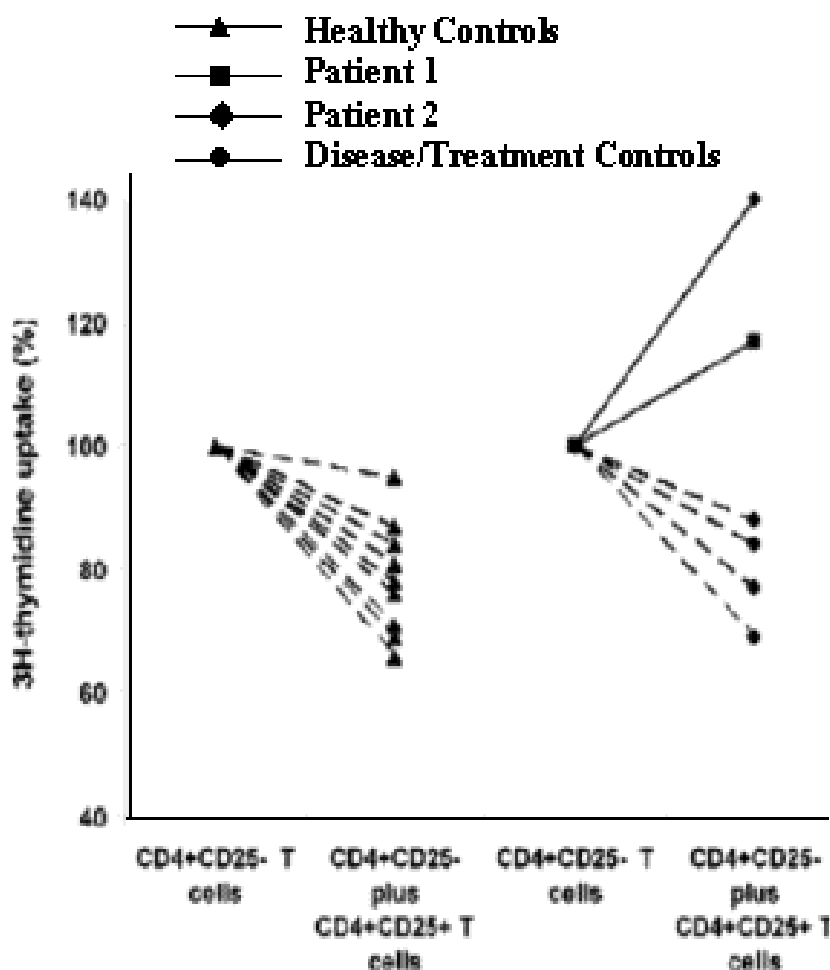


## 4.7 Phenotype and function of regulatory T cells

The 2 IPEX patients had a CD4<sup>+</sup> T cell subpopulation that constitutively expressed CD25. Flow cytometry analysis showed that the percentage of CD4<sup>+</sup>CD25<sup>+</sup> was normal compared with that found in healthy controls (Table 3). Children with other chronic autoimmune diseases who were treated with the same immune-suppressants had similarly normal percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells (Table 3). Treg cells are enriched in the population expressing high levels of CD25 (CD4<sup>+</sup>CD25<sup>bright</sup> T cells). Evaluation of CD4<sup>+</sup>CD25<sup>bright</sup> T cells showed no differences from healthy controls (Table 3). In both IPEX patients, similar to disease/treatment control patients, a negligible percentage of the CD4<sup>+</sup>CD25<sup>+</sup> T cells expressed the activation marker CD69 (Table 3), therefore suggesting that antigen-activated T cells did not contribute significantly to the subset of CD4<sup>+</sup>CD25<sup>+</sup> T cells. To investigate the function of the CD4<sup>+</sup>CD25<sup>+</sup> population, we evaluated the ability of CD4<sup>+</sup>CD25<sup>+</sup> to suppress the proliferation of autologous CD4<sup>+</sup>CD25<sup>+</sup> T cells. CD4<sup>+</sup>CD25<sup>+</sup> T cells from healthy controls suppressed the proliferation of autologous effector T cells by 15%–40% (Fig.12). On the contrary, co culture of CD4<sup>+</sup>CD25<sup>+</sup> T cells from IPEX patients with autologous T cells did not result in any suppression, but rather in increased proliferation (Fig.12). CD4<sup>+</sup>CD25<sup>+</sup> T cells from disease/treatment controls suppressed proliferation of autologous T cells in a range similar to that of healthy controls, excluding that the lack of suppression by the CD4<sup>+</sup>CD25<sup>+</sup> cells of IPEX patients was secondary to treatment (Fig. 12).

| <b>sample</b>                                                                                                                                                                                                                                                                                                                                                                                                                                            | <b>CD25<sup>+</sup> total<br/>(% CD4<sup>+</sup>)</b> | <b>CD25<sup>+</sup> bright<br/>(% CD4<sup>+</sup>)</b> | <b>CD69<sup>+</sup><br/>(%CD4<sup>+</sup>CD25<sup>+</sup>)</b> |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------|--------------------------------------------------------|----------------------------------------------------------------|
| <b>Healthy controls<br/>(range)</b>                                                                                                                                                                                                                                                                                                                                                                                                                      | <b>6.5 - 10.7</b>                                     | <b>.48 - 1.72</b>                                      | <b>.6 – 4.2</b>                                                |
| <b>IPEX (patient 1)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                  | <b>7.3</b>                                            | <b>.91</b>                                             | <b>1.95</b>                                                    |
| <b>IPEX (patient 2)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                  | <b>6.6</b>                                            | <b>1.01</b>                                            | <b>1.79</b>                                                    |
| <b>Autoimmune/treatment</b>                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                       |                                                        |                                                                |
| <b>Control 1</b>                                                                                                                                                                                                                                                                                                                                                                                                                                         | <b>8.53</b>                                           | <b>.62</b>                                             | <b>2.41</b>                                                    |
| <b>Control 2</b>                                                                                                                                                                                                                                                                                                                                                                                                                                         | <b>9.2</b>                                            | <b>1.99</b>                                            | <b>1.89</b>                                                    |
| <b>Control 3</b>                                                                                                                                                                                                                                                                                                                                                                                                                                         | <b>6.92</b>                                           | <b>1.05</b>                                            | <b>1.29</b>                                                    |
| <b>Control 4</b>                                                                                                                                                                                                                                                                                                                                                                                                                                         | <b>10.38</b>                                          | <b>2.40</b>                                            | <b>.51</b>                                                     |
| Autoimmune/treatment: control 1, systemic juvenile arthritis treated with prednisone (.4mg/kg/day) and cyclosporine A (4 mg/kg/day); control 2, juvenile dermatomyositis treated with cyclosporine A (5mg/kg/day); control 3, systemic juvenile idiopathic arthritis treated with prednisone (.1mg/kg/day) and cyclosporine A (5mg/kg/day); control 4; systemic lupus erythematosus treated with prednisone (.4mg/kg/day) and azathioprine (2mg/kg/day). |                                                       |                                                        |                                                                |

**Table 3.** CD4<sup>+</sup> CD25<sup>+</sup> T Cells in Patients with IPEX Syndrome, Healthy Controls, and in Patients with Autoimmune Diseases Treated with Glucocorticoids and Immune-suppressants



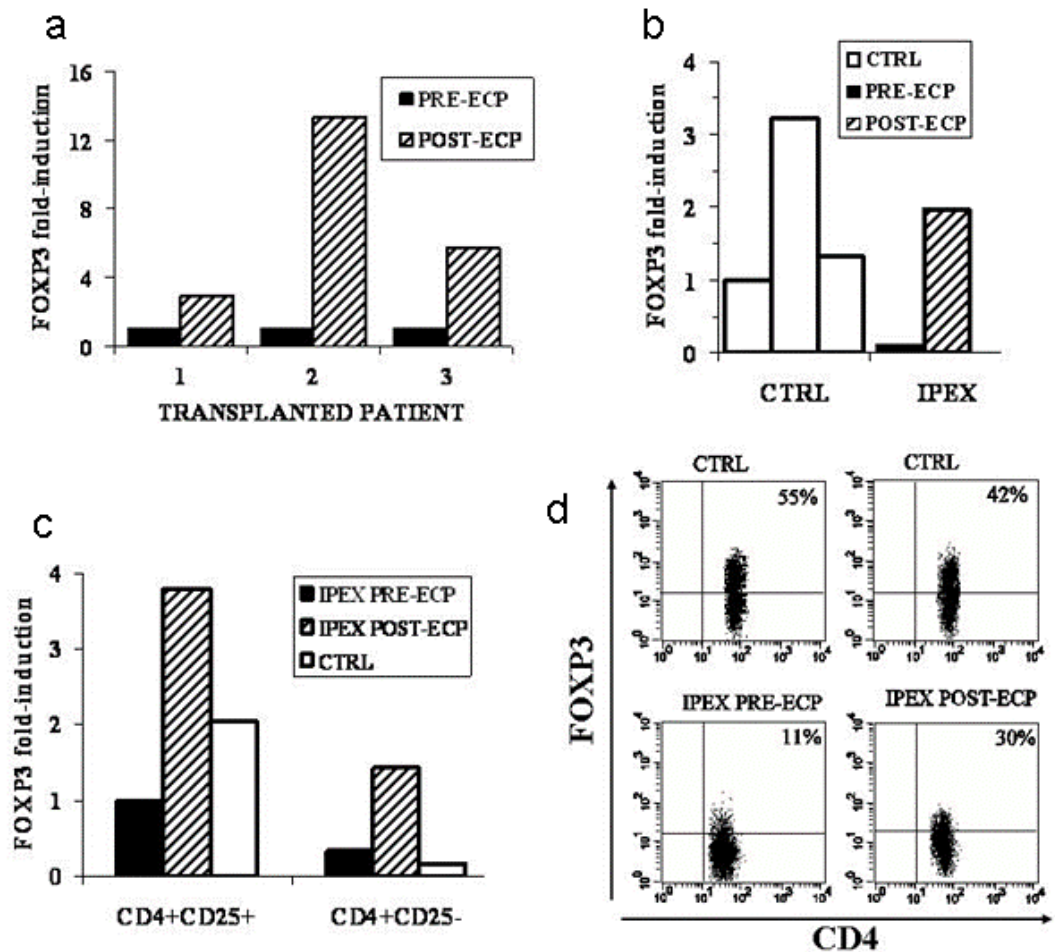
**Fig.12** Proliferation of effector  $CD4^+CD25^-$  T lymphocytes in the absence or in the presence of  $CD4^+CD25^+$  Treg from healthy controls, from autoimmune/treatment controls, and from the 2 IPEX patients.  $CD4^+CD25^-$  and  $CD4^+CD25^+$  populations were purified by sorting and were stimulated with anti-CD3 and anti-CD28 as described in the Materials and Methods section. Results are expressed as a percent of thymidine uptake of stimulated effector  $CD4^+CD25^-$  T lymphocytes cultured in the absence of  $CD4^+CD25^+$  T cells. (▲) Healthy controls; (■) patient 1 (◆) patient 2;; (●) disease/treatment controls.

## **4.8 ECP increases FOXP3 expression in CD4+ T lymphocytes**

Patient 1 showed a milder clinical phenotype and long-term survival. His FOXP3 splicing mutation (454+4A>G in intron 3) causes the generation of a splicing variant and the predicted protein lacks the fork-head domain, which is essential for FOXP3 function. We also found that this mutation allows the generation of low amounts of normal full length FOXP3 mRNA [64]. This genetic background, the poor control of clinical manifestations by conventional immune-suppression, and the previous observation that ECP treatment induces Treg *in vivo* [54] made us hypothesize that treatment with ECP in this patient could force FOXP3 expression inducing a significant increase in the amount of normal FOXP3 mRNA leading therefore to an increase in regulatory T cells and to a better control of the clinical manifestations. Incidentally, the primers used for the Real-time PCR assay amplify a 69bp amplicon which lies between exon 10 and 11 of the FOXP3 gene and code for the functional forkhead domain present only in full length FOXP3 mRNA and not in the abnormal splicing variant produced by the IPEX patient 1.

Indeed in preliminary experiments we found that treatment with ECP caused an increase in FOXP3 mRNA expression *in vivo* in otherwise healthy individuals who underwent organ transplantation (Fig.13a), therefore suggesting that the *in vivo* increase in regulatory T cells following ECP treatment involves induction of FOXP3 expression. As expected, before treatment with ECP normal full-length FOXP3 mRNA levels in the IPEX patient 1 were markedly lower than those of the healthy controls (Fig.13b). One month after beginning ECP, full-length FOXP3 mRNA

levels increased markedly to levels comparable to those of healthy controls (Fig.13b). The increase in full-length FOXP3 mRNA was present in the CD4<sup>+</sup>CD25<sup>+</sup> population, as well as in the CD4<sup>+</sup>CD25<sup>-</sup> T cell population (Fig.12c). Consistently, flow cytometry analysis revealed a significant increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells expressing the FOXP3 protein (Fig.13d). The latter observation suggests that ECP can induce *de novo* expression of FOXP3 in non-regulatory T cells.



**Fig.13 Extracorporeal photopheresis up-regulates FOXP3 expression.**

Normal full-length FOXP3 mRNA expression assessed by real-time PCR in: (a) PBMC from organ transplanted patients before (■) and 1 month after beginning ECP treatment (▨), (b) PBMC from healthy controls (□ n=3) and from the IPEX patient before (■) and 1 month after beginning ECP treatment (▨), (c) CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells purified by cell sorting from a healthy control (□) and from the IPEX patient before (■) and 1 month after beginning ECP treatment (▨). Results are shown relative to pre-treatment levels using the comparative C<sub>T</sub> method as described in Materials and Methods. (D) Intracellular FOXP3 protein expression by flow cytometry in CD4<sup>+</sup>CD25<sup>+</sup> T cells from two representative (out of 10) healthy controls and from the IPEX patient before and 1 month after beginning of ECP treatment.

## **4.9 ECP induces circulating CD4<sup>+</sup>CD25<sup>+</sup> T cells with regulatory function**

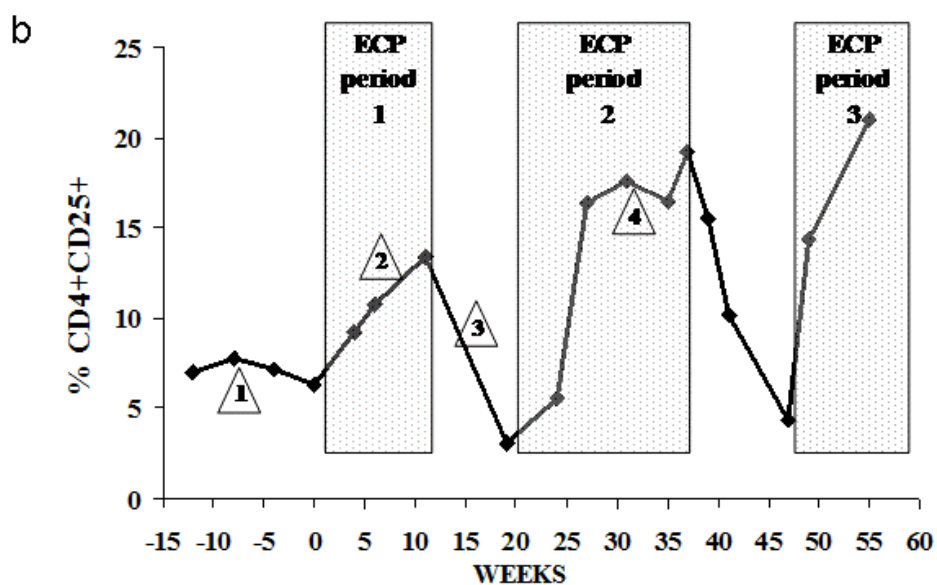
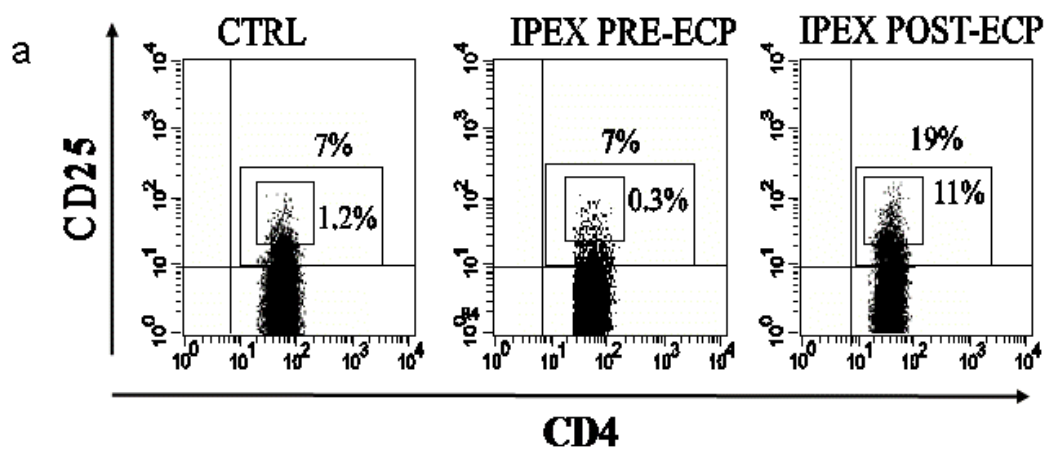
Flow cytometry analysis showed that the treatment with ECP induced in the IPEX patient 1 a substantial increase in the percentage of circulating CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>-</sup> T cells (Fig.14a). When CD4<sup>+</sup>CD25<sup>high</sup> CD69<sup>-</sup> T cells were evaluated, a marked increase was also observed (Fig.14a). The patient refused to follow a continuous every other week ECP treatment protocol, and therefore the treatment was administered in three different periods separated by interruptions of 6 to 8 weeks. We have sequentially measured the levels of CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>-</sup> T cells for 1 year. Following the beginning of each ECP treatment period we observed an increase in CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>-</sup> T cells (Fig.14b). During ECP the percentage of CD4<sup>+</sup>CD25<sup>+</sup> (mean  $20,2 \pm 10,1$ ) and of CD4<sup>+</sup>CD25<sup>high</sup> (mean  $8,9 \pm 2,2$ ) was significantly higher ( $p= 0.04$  and  $p= 0.006$ , respectively) than that observed when the patient was not treated with ECP (mean  $6,7 \pm 3$  and  $0,23 \pm 0,07$ , respectively).

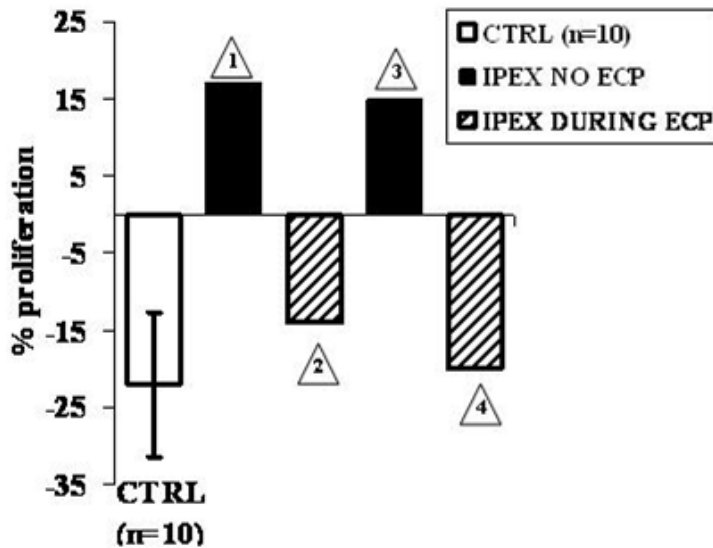
To verify whether the CD4<sup>+</sup>CD25<sup>+</sup> T cells induced by ECP treatment showed regulatory function, we evaluated the ability of CD4<sup>+</sup>CD25<sup>+</sup> T cells, present in the total CD4<sup>+</sup> T cell population, from the IPEX patient 1 to suppress the proliferation of activated autologous effector CD4<sup>+</sup>CD25<sup>-</sup> T cells stimulated with anti-CD3 and anti-CD28. CD4<sup>+</sup>CD25<sup>+</sup> T cells from healthy controls suppressed the proliferation of autologous effector T cells by 15%–40% (mean  $29,3 \pm 9,3$ ). As previously mentioned, CD4<sup>+</sup>CD25<sup>+</sup> T cells from patients with IPEX have defective suppressive function in vitro. Co-culture of CD4<sup>+</sup>CD25<sup>+</sup> T cells from IPEX patient 1 with autologous T cells before ECP treatment did not result in any suppression, but rather in

increased proliferation (Fig.14c). In contrast, following ECP treatment CD4<sup>+</sup>CD25<sup>+</sup> T cells from IPEX patient 1 suppressed proliferation of autologous effector CD4<sup>+</sup>CD25<sup>-</sup> T cells to an extent similar to that of healthy controls (Fig.14c). This suppressive activity was lost when ECP was suspended.

IPEX patient 1 was treated with three cycles of ECP (every two weeks for the first month, then every other week) as an add-on to standard treatment with prednisone, azathioprine and FK506 (Table 4). Following ECP treatments his clinical manifestations disappeared, renal function and metabolic acidosis normalized, a 5 Kg weight increase occurred, nightly parenteral nutrition was withdrawn and immunosuppressive treatment was tapered (Table 4). As previously mentioned the patient refused a continuous every other week ECP treatment protocol. Following the interruption of treatment intestinal symptoms reappeared together with a decrease in regulatory T cell numbers and a loss of suppressive function.







**Fig.14 ECP treatment increases CD4<sup>+</sup>CD25<sup>+</sup> with a regulatory function in the IPEX patient.** (a) Representative plots of CD4<sup>+</sup>CD25<sup>+</sup> T cells from the IPEX patient before and after ECP treatment. The percentage of total CD4<sup>+</sup>CD25<sup>+</sup> and of CD4<sup>+</sup>CD25<sup>high</sup> are indicated (b) Percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells during the different periods of ECP treatments, indicated by the shaded boxes. (c) The functional activity of CD4<sup>+</sup>CD25<sup>+</sup> T cells was evaluated by the ability to suppress proliferation of stimulated autologous CD4<sup>+</sup>CD25<sup>-</sup> effector T lymphocytes as described in Material and Methods. CD4<sup>+</sup>CD25<sup>-</sup> and total CD4<sup>+</sup> T cells, containing the CD4<sup>+</sup>CD25<sup>+</sup> T cell population, were purified by cell sorting from healthy controls ( $\square$  n=10) and from the IPEX patient 1 before ( $\blacksquare$ ) and during ( $\hatchedbox$ ) period 1 and 2 of ECP treatment. After 72 hours of culture, <sup>3</sup>H-thymidine incorporation was determined. Results are expressed as percent proliferation of stimulated effector CD4<sup>+</sup>CD25<sup>-</sup> T lymphocytes cultured in the presence of autologous total CD4<sup>+</sup> T cells.  $\triangle$  Indicate the period during which blood samples were collected.

|                                         | Enteropathy            | Skin           | Body weight | Treatment            |                      |                       |                |                       |
|-----------------------------------------|------------------------|----------------|-------------|----------------------|----------------------|-----------------------|----------------|-----------------------|
|                                         | Diarrhoea episodes/day | Severity score | (Kg)        | Parenteral nutrition | Oral PDN (mg/Kg/day) | MPDN pulses (n/month) | FK506 (mg/day) | Azathioprine (mg/day) |
| <b>Before ECP</b>                       | 9 ± 3                  | 3              | 47          | Yes                  | 1.1                  | 3                     | 4              | 100                   |
| <b>End of 1st ECP period</b>            | 0                      | 2              | 51          | No                   | 0.3                  | 0                     | 3              | 100                   |
| <b>Before 2<sup>nd</sup> ECP period</b> | 6 ± 2                  | 2              | 49          | Yes                  | 0.6                  | 3                     | 3              | 100                   |
| <b>End of 2<sup>nd</sup> ECP period</b> | 0                      | 0              | 54          | No                   | 0.2                  | 0                     | 3              | 50                    |
| <b>Before 3rd ECP period</b>            | 5 ± 3                  | 0              | 50          | Yes                  | 0.5                  | 3                     | 0              | 50                    |
| <b>End of 3<sup>rd</sup> ECP period</b> | 1 ± 2                  | 0              | 53          | No                   | 0.2                  | 0                     | 2              | 50                    |

**Table 4.** Treatment and clinical manifestations in IPEX patient 1 before and after each of the three ECP cycles.

## **5. DISCUSSION**

### **5.1 PAPA Syndrome**

The PAPA associated mutations in CD2BP1 do not affect its interaction with either CD2 or WASP [21], and up to now no defects in adaptive immunity have been reported in PAPA syndrome patients. The severe neutrophil infiltration in affected tissues of PAPA patients suggests a model of disease pathogenesis in which inflammation results directly from dysfunction of the innate immune response. In this scenario, disease-causing mutations in CD2BP1 somehow exaggerate the signal for proliferation and infiltration of inflammatory initiator cells, alter apoptotic pathways and/or inhibit their clearance, leading to the observed abnormal accumulations of neutrophil-rich material.

Our study of a subject with PAPA syndrome, of his affected mother and maternal uncle, the third family reported in the literature, support this idea. We show that PBMC isolated from the affected subjects produce markedly increased amounts of TNF $\alpha$  and treatment of the proband with the TNF inhibitor etanercept resulted in clinical long-lasting remission. This also underscores the possible role of TNF $\alpha$  in the inflammatory process of this disease. The high in vitro TNF $\alpha$  production of patients with genetically confirmed PAPA syndrome is consistent with this hypothesis.

At high concentrations, TNF $\alpha$  can lead to excess inflammation and organ injury. Activated macrophages are the primary source of TNF $\alpha$  in inflamed synovial tissue, and both the number of macrophages and the degrees of TNF $\alpha$  expression correlate with clinical scores for knee pain [65]. Acute

release of very large amounts of TNF $\alpha$  during sepsis may result in septic shock. In disease states, TNF $\alpha$  is generally considered to be a pro-inflammatory cytokine, along with IL-1 $\beta$ , IL-6, IL-17, and other cytokines. TNF $\alpha$  is a pleiotropic cytokine, in that it mediates a variety of direct pathogenic effects and induces the production of other mediators of inflammation and tissue destruction, placing it at the head of an inflammatory cascade within an inflammatory network. [66]. A variety of activities of TNF $\alpha$  such as cell recruitment, cell proliferation, cell death and immune regulation [66, 67] are thought to be inhibited by TNF antagonists [68].

As a result of tyrosine hyperphosphorylation, PAPA-associated CD2BP1 mutants bind with higher and stronger affinity pyrin, the protein encoded by the MEFV gene responsible for FMF [30]. This observation, together with our findings, suggest that mutation in CD2BP1 leads to deranged regulation of the inflammatory response. Indeed changes produced by CD2BP1 missense mutations are sufficient to confer susceptibility to inflammatory episodes, suggesting that CD2BP1 is critical in the homeostasis of inflammation. FMF is a rare, recessively inherited auto-inflammatory disease characterized by self-limited episodes of fever with arthritis, and sterile inflammation of peritoneal and pleural membranes [69]. Although clinically distinct from PAPA syndrome, FMF is also characterized by neutrophil infiltration into target tissues, and pyrin itself is expressed in neutrophils, but not T or B cells [70].

Pyrin binds to and inhibits ASC, a protein involved in recruitment and activation of caspase-1, which in turn is responsible for processing pro- IL-1 $\beta$  in its mature form [71]. Pyrin-deficient mice show increased IL-

1 $\beta$  processing with increased mature IL-1 $\beta$  release [72]. On the basis of these observations, it was hypothesized that CD2BP1 mutants sequester pyrin, causing a loss-of-function effect on the regulatory activity of pyrin. This ultimately leads to increased IL-1 $\beta$  processing. Indeed, Shoham et al observed that one patient with PAPA syndrome showed markedly increased IL-1 $\beta$  release after a short-term stimulation with LPS. We have confirmed this observation showing in 2 additional patients with PAPA syndrome that a short stimulation leads to a marked increase in IL-1 $\beta$  release. In addition, we have shown that PAPA-associated CD2BP1 mutation does not directly affect in vitro IL-1 $\beta$  production after a long-term stimulation (18 hours), involving gene expression, mRNA translation, and protein processing. On the other hand, we have shown that individuals with PAPA syndrome produce high amounts of TNF- $\alpha$  after an 18-hour stimulation and that this increased production can only partially be explained by the early release of IL-1 $\beta$ . The latter findings suggest that CD2BP1 mutations may affect inflammatory cytokine production through different pathways.

These findings may again be partially explained by the interactions with pyrin and ASC. Indeed, ASC has been shown not only to mediate caspase-1 recruitment and activation but also to modulate NF- $\kappa$ B signalling pathways, suggesting broader modulatory effects on inflammatory responses [73, 74]. High production of IL-6, TNF $\alpha$ , and IL-8 has been reported in attack-free patients with Familial Mediterranean Fever, supporting the hypothesis for a role of pyrin and ASC in the modulation of several inflammatory cytokines, in addition to IL-1 $\beta$  [75].

The perturbed pattern of priming/endotoxin tolerance showed for IL-6 may support this idea. PBMC from the PAPA patients, unlike those from

healthy individuals or oligo-JIA patients, failed to reduce IL-6 production after a high dose of LPS pre-treatment, indicating that another pro-inflammatory actor may be involved in this complex scenario. The proband also showed a high priming response to low doses of LPS. It is conceivable that the altered endotoxin priming/tolerance pattern for IL-6 may reflect the perturbed regulation of inflammatory response and at least in part explain the hyper-responsiveness of inflammatory cells and the low-level inflammation constitutively present in PAPA syndrome. Interestingly clinical flares in PAPA patients may result from mild physical trauma, suggesting abnormal response to otherwise non pathogenic stimuli [76].

No definitive treatment is known for this disease and management of PAPA syndrome represents a challenge. Our patient, after an initial response, had resistance to glucocorticoids with recurrences on a daily regimen, therefore showing that failure to respond satisfactorily to glucocorticoids may occur in PAPA syndrome. The laboratory findings provided an additional background for administration of the TNF inhibitor etanercept. The observation that this anti-TNF agent was associated with a rapid, sustained clinical remission suggests that etanercept represents a valuable therapeutic option in PAPA patients with unsatisfactory response to glucocorticoids.

## **5.2 IPEX Syndrome**

The two IPEX patients we have studied appear to have a milder course disease than that of other IPEX patients reported. Both our patients survived the early years of life and patient 1 reached young adulthood. In both

patients early onset severe enteropathy was the prominent clinical feature. In addition, our patients did not develop clinical or subclinical (presence of auto-antibodies) evidence of T1D or other autoimmune endocrinopathies, or severe or unusual infections despite chronic immune-suppression. To date, the majority of FOXP3 mutations reported in IPEX patients are either missense mutations in the forkhead DNA binding domain, or deletions resulting in an altered, truncated, or missing forkhead domain [7, 50].

In patient 2, a missense mutation in exon 3 leads to an amino-acid substitution that does not affect the forkhead domain. In patient 1, the mutation close to the splice site generates abnormal and normal transcripts, suggesting that at least a certain amount of normal FOXP3 protein is produced. This phenomenon (i.e., splicing mutations resulting in abnormal and some normal transcripts), usually not reported in IPEX, already has been described in X-linked adrenoleukodystrophy [77]. Therefore it is tempting to speculate that a milder IPEX phenotype may be associated with FOXP3 mutations that do not abrogate the function of the forkhead domain. Further studies of genotype/phenotype correlation are needed. Interestingly, one of the few FOXP3 mutation associated with a mild IPEX phenotype reported to date is that of the family described by Powell et al [47], which affected the first polyadenylation signal, possibly resulting in messenger RNA destabilization and in reduced production of a normal protein [78].

In mice, FOXP3 has been shown to be essential for the development and function of Treg cells. No data were available on the function of Treg cells in the patients with IPEX. The percentage and absolute number of circulating CD4<sup>+</sup>CD25<sup>+</sup> T cells were in the low-normal range in both our patients. Because only a negligible percentage of these cells expressed the activation marker CD69, these CD4<sup>+</sup>CD25<sup>+</sup> T cells are unlikely to represent



activated T cells. We found that the CD4<sup>+</sup>CD25<sup>+</sup> T cells of both our patients were unable to perform a typical function of Treg cells in vitro: suppression of proliferation of activated T cells. Because anergy (absence of response to T-cell receptor cross-linking) is another feature of cultured Treg cells, the increased proliferation in cultures containing CD4<sup>+</sup>CD25<sup>+</sup> from our IPEX patients suggests lack of anergy. Therefore, the CD4<sup>+</sup>CD25<sup>+</sup> cells from our patients have a functional impairment, being both unable to suppress and to remain anergic. Taken together, our findings suggest that, even if not affecting the forkhead domain, these novel FOXP3 mutations are relevant clinically and functionally and imply that other domains, besides the forkhead domain, play a role in the FOXP3 function. In an interesting article by Bacchetta et al. [79] a clear correlation is also made between mutations in different discrete domains of FOXP3 with the number, phenotype and function of Treg in 4 IPEX patients with different clinical phenotypes.

The peculiar mutation of Patient 1 (a splicing mutation allowing the generation of low amounts of normal full length FOXP3 mRNA), the poor control of clinical manifestations by conventional immune-suppression, and the previous observation that ECP treatment induces Treg in vivo [54], made us hypothesize that treatment with ECP in this patient could force FOXP3 expression. This would in turn induce a significant increase in the amount of normal FOXP3 mRNA leading to an increase in regulatory T cells and possibly to a better control of the clinical manifestations. The treatment with ECP of IPEX patient 1 induced an increase in regulatory T cells with suppressive function in vitro. This was associated with a significant clinical improvement allowing tapering of immunosuppressive treatments, significant amelioration of symptoms and discontinuation of parenteral nutrition. The correlation between treatment periods, increase in

regulatory T cells and clinical response strongly support a causal relationship.

Although the mechanism by which ECP exerts its therapeutic benefits in immune disorders is not completely clarified, induction of CD4<sup>+</sup>CD25<sup>+</sup> Treg may play a role [1]. Indeed, UV-induced Treg belong to the CD4<sup>+</sup>CD25<sup>+</sup> subtype [80] and express CTLA-4 [81]. In our patient, ECP appears to induce peripheral Treg. This is supported by a) the age of the patient implying little, if any, residual thymic activity b) after ECP we observed markedly increased expression of FOXP3 mRNA, not only in the CD4<sup>+</sup>CD25<sup>+</sup> T cell population, but also in CD4<sup>+</sup>CD25<sup>-</sup> T cells. It is tempting to speculate that these cells are being driven towards the acquisition of a Treg phenotype.

The increase in the levels of CD4<sup>+</sup>CD25<sup>+</sup> T cells was greater in each of the consecutive periods of ECP treatment and the increase was gradual during the first ECP treatment and rather sharp during the second and third period. This reminds of a booster (recall) effect. The biological mechanisms underlying this observation awaits to be unravelled. In both instances in which ECP treatment was suspended, a rapid decrease in regulatory T cell number occurred, associated with a rather rapid appearance of symptoms. In kidney transplanted patients we have recently observed that following a short period of ECP treatments (6 procedures in 3 weeks) the increase in regulatory T cells persisted for as long as one year [82]. The discrepancy with the IPEX patient may be explained by the fact that the kidney transplanted patients have a normal FOXP3 gene; in contrast, the FOXP3 mutation carried by our patient allows only the expression of low amounts of normal transcript, suggesting that continuous stimulation is necessary to maintain sufficient levels of normal FOXP3 mRNA and of regulatory T cell

activity. This hypothesis is consistent with the observation that in mice FOXP3 function promotes its own transcription and FOXP3 transcription is not maintained in the absence of functional FOXP3 protein [83]. In our patient suspension of ECP led also to clinical manifestations. This observation, as well as the past clinical history of the patient, show that even if a mutation allows formation of some normal FOXP3 transcript, this is not sufficient to maintain homeostasis. Indeed, recent studies in mice showed that attenuated (5-10 fold) expression of FOXP3 caused a clinically relevant autoimmune disorder, which was less severe than that observed in the original FOXP3 knock-out mice. Also in mice it has been shown that decreased FOXP3 expression can caused defective suppressive function of Treg cells and their conversion into effector cells, which contribute to rather than inhibit immune diseases [84]. This leads to the conclusion that FOXP3 may not serve as an on-and-off trigger of the development and activity of regulatory T cells, but rather functions in a dose-dependant manner.

In summary we have reported 2 patients with IPEX presenting with early onset enteropathy without T1D or other endocrinopathies. These patients carry novel mutations of the FOXP3 gene that do not abrogate the function of the forkhead domain. Nevertheless they have functionally defective Treg cells. Our results with ECP treatment showed that ECP may represent a valid therapeutic approach for IPEX patients, carrying FOXP3 mutations that do not completely abrogate FOXP3 expression and function. It may also be envisaged that ECP could be used as a bridge treatment to obtain better disease control and better clinical conditions before bone marrow transplantation [85]. Beside IPEX syndrome, several observations in humans and animals are consistent with the hypothesis that subtle defects in Treg activity may be important in the pathogenesis of autoimmune diseases

[86]. ECP may represent a tool to investigate whether an increase in regulatory T cell activity is related to therapeutic benefit in autoimmunity or other immune conditions such as GVHD and organ rejection.

## **6. CONCLUSION**

The work presented involved a close collaboration between clinical researchers and laboratory researchers. Both our studies on the PAPA and IPEX syndrome, although different pathologies affecting different cell types and inflammatory mediators, demonstrate how the characterization of the cellular and molecular abnormalities and of the subsequent diseases mechanisms provided a rational for the pianification of therapeutic approaches. The close collaboration between clinical and laboratory immunologists allowed a rapid translation of laboratory acquisition into the development of therapeutic tools for disease-specific intervention.

## **7. ABBREVIATIONS**

PAPA syndrome: pyogenic sterile arthritis, pyoderma gangrenosum, acne

CD2BP1: CD2 binding protein 1

IDDM : insuline-dependent diabetes mellitus

T1D: type 1 diabetes

PSTPIP1: phosphatase- proline serine threonine interacting protein 1

PTPs: PEST-type protein tyrosine phosphatases

WASP: Wiskott Aldrich syndrome protein

TNF-  $\alpha$ : tumor necrosis factor-  $\alpha$

IL-1 $\beta$ : interleukin-1

TGF $\beta$ : Tumor growth factor  $\beta$

IL-6: interleukine-6

HPFs: hereditary periodic fever syndromes

LPS: lipopolysaccharide

PAMPs: pathogen-associated molecular patterns

TLRs: Toll-like receptors

FMF: familial Mediterranean fever

TRAPS: TNF-receptor-associated periodic syndrome

FCAS: familial cold autoinflammatory syndrome

CINCA: chronic infantile neurological cutaneous and articular syndrome

NOMID: neonatal onset multisystemic inflammatory disease

Oligo-JIA: oligoarticular juvenile idiopathic arthritis

IPEX syndrome: Immunodysregulation, Polyendocrinopathy,  
Enteropathy, X-linked

APECED: autoimmune polyendocrinopathy candidiasis ectodermal  
dystrophy

FOXP3: transcription factor forkhead box protein 3

Treg: regulatory T cells

ECP: extracorporeal photopheresis

CTLA4: CTL-associated antigen 4

GITR: glucocorticoid-induced TNF receptor

NFAT: nuclear factor of activated T cells

NF-κB: nuclear factor-κB

DCs: dendritic cells

FKH: forkhead domain

ANAs: antinuclear antibodies

NOD-LRR: nucleotide-binding oligomerization domain-leucine rich repeat  
proteins

ESR: Erythrocyte sedimentation rate

CRP: C-reactive protein;

WBC: white blood cell;

ND: not determined

## **8. REFERENCES**

1. Lamioni, A., et al., *The immunological effects of extracorporeal photopheresis unraveled: induction of tolerogenic dendritic cells in vitro and regulatory T cells in vivo*. Transplantation, 2005. **79**(7): p. 846-50.
2. Maeda, A., *Intravenous infusion of syngeneic apoptotic cells by photopheresis induces antigen-specific regulatory T cells*. J Immunol, 2005. **174**(10): p. 5968-76.
3. Jacobson, D.L., et al., *Epidemiology and estimated population burden of selected autoimmune diseases in the United States*. Clin Immunol Immunopathol, 1997. **84**(3): p. 223-43.
4. Davidson, A. and B. Diamond, *Autoimmune diseases*. N Engl J Med, 2001. **345**(5): p. 340-50.
5. Sneller, M.C., J.K. Dale, and S.E. Straus, *Autoimmune lymphoproliferative syndrome*. Curr Opin Rheumatol, 2003. **15**(4): p. 417-21.
6. Halonen, M., et al., *AIRE mutations and human leukocyte antigen genotypes as determinants of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy phenotype*. J Clin Endocrinol Metab, 2002. **87**(6): p. 2568-74.
7. Gambineri, E., T.R. Torgerson, and H.D. Ochs, *Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis*. Curr Opin Rheumatol, 2003. **15**(4): p. 430-5.
8. Galon, J., et al., *TNFRSF1A mutations and autoinflammatory syndromes*. Curr Opin Immunol, 2000. **12**(4): p. 479-86.
9. Herbeden, W., *Commentaries on History and Care of Disease*, in London:T. Payne. 1802, London:T. Payne p. p. 151.
10. McDermott, M.F., *A common pathway in periodic fever syndromes*. Trends Immunol, 2004. **25**(9): p. 457-60.
11. Simon, A. and J.W. van der Meer, *Pathogenesis of familial periodic fever syndromes or hereditary autoinflammatory syndromes*. Am J Physiol Regul Integr Comp Physiol, 2007. **292**(1): p. R86-98.
12. McDermott, M.F., et al., *Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of*

- dominantly inherited autoinflammatory syndromes.* Cell, 1999. **97**(1): p. 133-44.
13. Tunca, M. and H. Ozdogan, *Molecular and genetic characteristics of hereditary autoinflammatory diseases.* Curr Drug Targets Inflamm Allergy, 2005. **4**(1): p. 77-80.
  14. Romanovsky, A.A., et al., *Fever and hypothermia in systemic inflammation: recent discoveries and revisions.* Front Biosci, 2005. **10**: p. 2193-216.
  15. Dinarello, C.A., *Unraveling the NALP-3/IL-1beta inflammasome: a big lesson from a small mutation.* Immunity, 2004. **20**(3): p. 243-4.
  16. Steiner, A.A., et al., *Bacterial lipopolysaccharide fever is initiated via Toll-like receptor 4 on hematopoietic cells.* Blood, 2006. **107**(10): p. 4000-2.
  17. Creagh, E.M. and L.A. O'Neill, *TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity.* Trends Immunol, 2006. **27**(8): p. 352-7.
  18. Dinarello, C.A., *Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed.* J Endotoxin Res, 2004. **10**(4): p. 201-22.
  19. Gabay, C. and I. Kushner, *Acute-phase proteins and other systemic responses to inflammation.* N Engl J Med, 1999. **340**(6): p. 448-54.
  20. Lindor, N.M., et al., *A new autosomal dominant disorder of pyogenic sterile arthritis, pyoderma gangrenosum, and acne: PAPA syndrome.* Mayo Clin Proc, 1997. **72**(7): p. 611-5.
  21. Wise, C.A., et al., *Mutations in CD2BP1 disrupt binding to PTP PEST and are responsible for PAPA syndrome, an autoinflammatory disorder.* Hum Mol Genet, 2002. **11**(8): p. 961-9.
  22. Yeon, H.B., et al., *Pyogenic arthritis, pyoderma gangrenosum, and acne syndrome maps to chromosome 15q.* Am J Hum Genet, 2000. **66**(4): p. 1443-8.
  23. Jacobs, J.C. and E.J. Goetzel, *"Streaking leukocyte factor," arthritis, and pyoderma gangrenosum.* Pediatrics, 1975. **56**(4): p. 570-8.
  24. Badour, K., J. Zhang, and K.A. Siminovitch, *The Wiskott-Aldrich syndrome protein: forging the link between actin and cell activation.* Immunol Rev, 2003. **192**: p. 98-112.
  25. Cote, J.F., et al., *PSTPIP is a substrate of PTP-PEST and serves as a scaffold guiding PTP-PEST toward a specific dephosphorylation of WASP.* J Biol Chem, 2002. **277**(4): p. 2973-86.



26. Cong, F., et al., *Cytoskeletal protein PSTPIP1 directs the PEST-type protein tyrosine phosphatase to the c-Abl kinase to mediate Abl dephosphorylation*. Mol Cell, 2000. **6**(6): p. 1413-23.
27. Spencer, S., et al., *PSTPIP: a tyrosine phosphorylated cleavage furrow-associated protein that is a substrate for a PEST tyrosine phosphatase*. J Cell Biol, 1997. **138**(4): p. 845-60.
28. Li, J., et al., *A cdc15-like adaptor protein (CD2BP1) interacts with the CD2 cytoplasmic domain and regulates CD2-triggered adhesion*. Embo J, 1998. **17**(24): p. 7320-36.
29. Fankhauser, C., et al., *The S. pombe cdc15 gene is a key element in the reorganization of F-actin at mitosis*. Cell, 1995. **82**(3): p. 435-44.
30. Shoham, N.G., et al., *Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway*. Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13501-6.
31. Papin, S., et al., *The SPRY domain of Pyrin, mutated in familial Mediterranean fever patients, interacts with inflammasome components and inhibits proIL-1beta processing*. Cell Death Differ, 2007. **14**(8): p. 1457-66.
32. Ziegler, S.F., *FOXP3: not just for regulatory T cells anymore*. Eur J Immunol, 2007. **37**(1): p. 21-3.
33. DeJaco, C., et al., *Imbalance of regulatory T cells in human autoimmune diseases*. Immunology, 2006. **117**(3): p. 289-300.
34. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases*. J Immunol, 1995. **155**(3): p. 1151-64.
35. Sullivan, K.E., D. McDonald-McGinn, and E.H. Zackai, *CD4(+) CD25(+) T-cell production in healthy humans and in patients with thymic hypoplasia*. Clin Diagn Lab Immunol, 2002. **9**(5): p. 1129-31.
36. Bensinger, S.J., et al., *Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells*. J Exp Med, 2001. **194**(4): p. 427-38.
37. Fontenot, J.D. and A.Y. Rudensky, *A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3*. Nat Immunol, 2005. **6**(4): p. 331-7.
38. von Boehmer, H., *Dynamics of suppressor T cells: in vivo veritas*. J Exp Med, 2003. **198**(6): p. 845-9.

39. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3*. Science, 2003. **299**(5609): p. 1057-61.
40. Ziegler, S.F., *FOXP3: of mice and men*. Annu Rev Immunol, 2006. **24**: p. 209-26.
41. Allan, S.E., et al., *The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs*. J Clin Invest, 2005. **115**(11): p. 3276-84.
42. Bettelli, E., M. Dastrange, and M. Oukka, *Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells*. Proc Natl Acad Sci U S A, 2005. **102**(14): p. 5138-43.
43. Schubert, L.A., et al., *Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation*. J Biol Chem, 2001. **276**(40): p. 37672-9.
44. Lopes, J.E., et al., *Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor*. J Immunol, 2006. **177**(5): p. 3133-42.
45. Ono, M., et al., *Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1*. Nature, 2007. **446**(7136): p. 685-9.
46. Li, B., et al., *FOXP3 ensembles in T-cell regulation*. Immunol Rev, 2006. **212**: p. 99-113.
47. Powell, B.R., N.R. Buist, and P. Stenzel, *An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy*. J Pediatr, 1982. **100**(5): p. 731-7.
48. Brunkow, M.E., et al., *Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse*. Nat Genet, 2001. **27**(1): p. 68-73.
49. Mirakian, R., et al., *Protracted diarrhoea of infancy: evidence in support of an autoimmune variant*. Br Med J (Clin Res Ed), 1986. **293**(6555): p. 1132-6.
50. Wildin, R.S., S. Smyk-Pearson, and A.H. Filipovich, *Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome*. J Med Genet, 2002. **39**(8): p. 537-45.
51. Torgerson, T.R., et al., *Severe food allergy as a variant of IPEX syndrome caused by a deletion in a noncoding region of the FOXP3 gene*. Gastroenterology, 2007. **132**(5): p. 1705-17.
52. Nieves, D.S., et al., *Dermatologic and immunologic findings in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome*. Arch Dermatol, 2004. **140**(4): p. 466-72.

53. Marshall, S.R., *Technology insight: ECP for the treatment of GvHD-can we offer selective immune control without generalized immunosuppression?* Nat Clin Pract Oncol, 2006. **3**(6): p. 302-14.
54. Aubin, F. and C. Mousson, *Ultraviolet light-induced regulatory (suppressor) T cells: an approach for promoting induction of operational allograft tolerance?* Transplantation, 2004. **77**(1 Suppl): p. S29-31.
55. Bladon, J. and P.C. Taylor, *Extracorporeal photopheresis in cutaneous T-cell lymphoma and graft-versus-host disease induces both immediate and progressive apoptotic processes.* Br J Dermatol, 2002. **146**(1): p. 59-68.
56. Di Renzo, M., et al., *ECP-treated lymphocytes of chronic graft-versus-host disease patients undergo apoptosis which involves both the Fas/FasL system and the Bcl-2 protein family.* Arch Dermatol Res, 2003. **295**(5): p. 175-82.
57. Voll, R.E., et al., *Immunosuppressive effects of apoptotic cells.* Nature, 1997. **390**(6658): p. 350-1.
58. Savill, J., et al., *A blast from the past: clearance of apoptotic cells regulates immune responses.* Nat Rev Immunol, 2002. **2**(12): p. 965-75.
59. Kunzmann, S., et al., *SARA and Hgs attenuate susceptibility to TGF-beta1-mediated T cell suppression.* Faseb J, 2003. **17**(2): p. 194-202.
60. Kobayashi, K., et al., *IRAK-M is a negative regulator of Toll-like receptor signaling.* Cell, 2002. **110**(2): p. 191-202.
61. Nomura, F., et al., *Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression.* J Immunol, 2000. **164**(7): p. 3476-9.
62. Peck, O.M., et al., *Differential regulation of cytokine and chemokine production in lipopolysaccharide-induced tolerance and priming.* Cytokine, 2004. **26**(5): p. 202-8.
63. Oberhuber, G., G. Granditsch, and H. Vogelsang, *The histopathology of coeliac disease: time for a standardized report scheme for pathologists.* Eur J Gastroenterol Hepatol, 1999. **11**(10): p. 1185-94.
64. De Benedetti, F., et al., *Mechanistic associations of a mild phenotype of immunodysregulation, polyendocrinopathy, enteropathy, x-linked syndrome.* Clin Gastroenterol Hepatol, 2006. **4**(5): p. 653-9.
65. Tak, P.P., et al., *Decrease in cellularity and expression of adhesion molecules by anti-tumor necrosis factor alpha monoclonal antibody*

- treatment in patients with rheumatoid arthritis. Arthritis Rheum*, 1996. **39**(7): p. 1077-81.
66. Feldmann, M., *Development of anti-TNF therapy for rheumatoid arthritis. Nat Rev Immunol*, 2002. **2**(5): p. 364-71.
  67. Schottelius, A.J., et al., *Biology of tumor necrosis factor-alpha-implications for psoriasis. Exp Dermatol*, 2004. **13**(4): p. 193-222.
  68. Choy, E. and G. Panayi, *Mechanisms of action of second-line agents and choice of drugs in combination therapy. Clin Exp Rheumatol*, 1999. **17**(6 Suppl 18): p. S20-8.
  69. Hull, K.M., et al., *The expanding spectrum of systemic autoinflammatory disorders and their rheumatic manifestations. Curr Opin Rheumatol*, 2003. **15**(1): p. 61-9.
  70. Chae, J.J., et al., *The B30.2 domain of pyrin, the familial Mediterranean fever protein, interacts directly with caspase-1 to modulate IL-1beta production. Proc Natl Acad Sci U S A*, 2006. **103**(26): p. 9982-7.
  71. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell*, 2002. **10**(2): p. 417-26.
  72. Chae, J.J., et al., *Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. Mol Cell*, 2003. **11**(3): p. 591-604.
  73. Stehlik, C., et al., *The PAAD/PYRIN-family protein ASC is a dual regulator of a conserved step in nuclear factor kappaB activation pathways. J Exp Med*, 2002. **196**(12): p. 1605-15.
  74. Stehlik, C., et al., *The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-kappa B and procaspase-1 regulation. Biochem J*, 2003. **373**(Pt 1): p. 101-13.
  75. Notarnicola, C., et al., *Enhanced cytokine mRNA levels in attack-free patients with familial Mediterranean fever. Genes Immun*, 2002. **3**(1): p. 43-5.
  76. Stichweh, D.S., M. Punaro, and V. Pascual, *Dramatic improvement of pyoderma gangrenosum with infliximab in a patient with PAPA syndrome. Pediatr Dermatol*, 2005. **22**(3): p. 262-5.
  77. Guimaraes, C.P., C. Sa-Miranda, and J.E. Azevedo, *Probing substrate-induced conformational alterations in adrenoleukodystrophy protein by proteolysis. J Hum Genet*, 2005. **50**(2): p. 99-105.
  78. Bennett, C.L. and H.D. Ochs, *IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy,*

- enteropathy, and a variety of autoimmune phenomena. Curr Opin Pediatr*, 2001. **13**(6): p. 533-8.
79. Bacchetta, R., et al., *Defective regulatory and effector T cell functions in patients with FOXP3 mutations. J Clin Invest*, 2006. **116**(6): p. 1713-22.
  80. Schwarz, A., et al., *Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. J Immunol*, 2004. **172**(2): p. 1036-43.
  81. Schwarz, A., et al., *Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. J Immunol*, 2000. **165**(4): p. 1824-31.
  82. Lamioni, A., et al., *Induction of regulatory T cells after prophylactic treatment with photopheresis in renal transplant recipients. Transplantation*, 2007. **83**(10): p. 1393-6.
  83. Gavin, M.A., et al., *Foxp3-dependent programme of regulatory T-cell differentiation. Nature*, 2007. **445**(7129): p. 771-5.
  84. Wan, Y.Y. and R.A. Flavell, *Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature*, 2007. **445**(7129): p. 766-70.
  85. Rao, A., et al., *Successful bone marrow transplantation for IPEX syndrome after reduced-intensity conditioning. Blood*, 2007. **109**(1): p. 383-5.
  86. Raimondi, G., et al., *Naturally occurring regulatory T cells: recent insights in health and disease. Crit Rev Immunol*, 2007. **27**(1): p. 61-95.

## **9. Publications and Posters**

De Benedetti F, Insalaco A, Diamanti A, Cortis E, **Muratori F**, Lamioni A, Carsetti R, Cusano R, De Vito R, Perroni L, Gambarara M, Castro M, Bottazzo GF, Ugazio AG. Mechanistic associations of a mild phenotype of immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Clin Gastroenterol Hepatol. 2006 May;4(5):653-9.

Cortis E, De Benedetti F, Insalaco A, Cioschi S, **Muratori F**, D'Urbano LE, Ugazio AG. Abnormal production of tumor necrosis factor (TNF)-alpha and clinical efficacy of the TNF inhibitor etanercept in a patient with PAPA syndrome. J Pediatr. 2004 Dec;145(6):851-5.

**Muratori F** et al. ECP Induces FOXP3 mRNA expression and regulatory T cells: a proof of principle in a patient with IPEX syndrome. Submitted

**F. Muratori**<sup>1</sup>, A. Lamioni<sup>2</sup>, M. Vivarelli<sup>1</sup>, A. Insalaco<sup>1</sup>, E. Cortis<sup>1</sup>, A. Landolfo<sup>3</sup>, F. De Benedetti<sup>1</sup>, A. G. Ugazio<sup>1</sup>. EXTRACORPOREAL PHOTOPHERESIS (ECP) AND INDUCTION OF REGULATORY T CELLS: A LESSON FROM IPEX SYNDROME. European league against rheumatism. Amsterdam 21-24 June 2006. Annals of the Rheumatic Diseases, 2006;65(supp. II): 248.

# ABNORMAL PRODUCTION OF THE TUMOR NECROSIS FACTOR INHIBITOR ETANERCEPT AND CLINICAL EFFICACY OF TUMOR IN A PATIENT WITH PAPA SYNDROME

ELISABETTA CORTIS, MD, FABRIZIO DE BENEDETTI, MD, ANTONELLA INSALACO, MD, STEFANIA CIOSCHI, MD, FLAMINIA MURATORI, PhD, LEILA E. D'URBANO, PhD, AND ALBERTO G. UGAZIO, MD

We report a family with pyogenic sterile arthritis, pyoderma and acne syndrome (PAPA). The proband presented several episodes of sterile pyogenic arthritis and became unresponsive to glucocorticoids. After treatment with the tumor necrosis factor inhibitor etanercept, the disease underwent rapid and sustained clinical remission. Production of tumor necrosis factor- $\alpha$  by mononuclear cells of the proband and of the affected relatives was abnormally elevated. (*J Pediatr* 2004;145:851-5)

**P** yogenic sterile arthritis, pyoderma and acne (PAPA) syndrome (OMIM No. 604410) is a rare autosomic dominant inherited autoinflammatory disorder of childhood, primarily affecting the joints and skin. The joint involvement is represented by destructive recurrent arthritis. Synovial fluid is purulent with neutrophil accumulation, but cultures are invariably negative. Arthritis and skin lesions have been reported to be responsive to glucocorticoids.<sup>1,2</sup>

To date, fewer than 10 patients with PAPA syndrome have been identified from two families.<sup>2,3</sup> Two mutations in the CD2 binding protein 1 (CD2BP1) gene have been associated with the disease.<sup>3</sup> The CD2BP1 gene encodes for an adaptor protein known to interact with phosphatases. Recently, the CD2BP1 protein has been demonstrated to bind pyrin, the protein coded by the MEFV gene and whose mutations cause familial mediterranean fever; PAPA-associated CD2BP1 mutants exhibit increased binding to pyrin.<sup>4</sup> Given the role of pyrin in regulating proinflammatory cytokine production, it is conceivable that CD2BP-1 affects inflammatory responses through modulation of inflammatory cytokines. However, the proinflammatory mediators involved in PAPA syndrome are still to be identified.

## METHODS

### Mutation Analysis

CD2BP1 exons were amplified from genomic DNA through the use of oligonucleotide primers reported by Wise et al.<sup>3</sup> Screening for mutations was performed by denaturing high-performance liquid chromatography (DHPLC) with the use of Wave analysis (Transgenomic, San Jose, Calif), using conditions recommended by WaveMaker 2.0 software. Polymerase chain reaction amplimers were purified with the Nucleospin kit (Macherey-Nagel, Duran, Germany) and sequenced with CEQ DTCS (Beckman, Fullerton, Calif), with the use of a CEQ 2000 automated sequencer (Beckman).

### In Vitro Cytokine Production

Peripheral blood mononuclear cells (PBMC) from 15 healthy control subjects (age range, 6 to 21 years), 15 patients with oligoarticular juvenile idiopathic arthritis (JIA) (age range, 4 to 18 years), and 3 unaffected and 3 affected individuals of the family with PAPA syndrome (Figure 1) were separated by means of Ficoll/Histopaque. Three samples of individual IV-2, one obtained before etanercept treatment, were available;  $2 \times 10^5$  PBMC were cultured in RPMI 1640 (GIBCO, Invitrogen Corp, Carlsbad, Calif) with 10% FBS (HyClone, Logan, Utah) at 37°C in 5% CO<sub>2</sub> in a 96-well plate with or without 10 ng/mL

From the Department of Medicine, Division of Rheumatology and Laboratory of Rheumatology, IRCCS Ospedale Pediatrico Bambino Gesù, Rome, Italy.

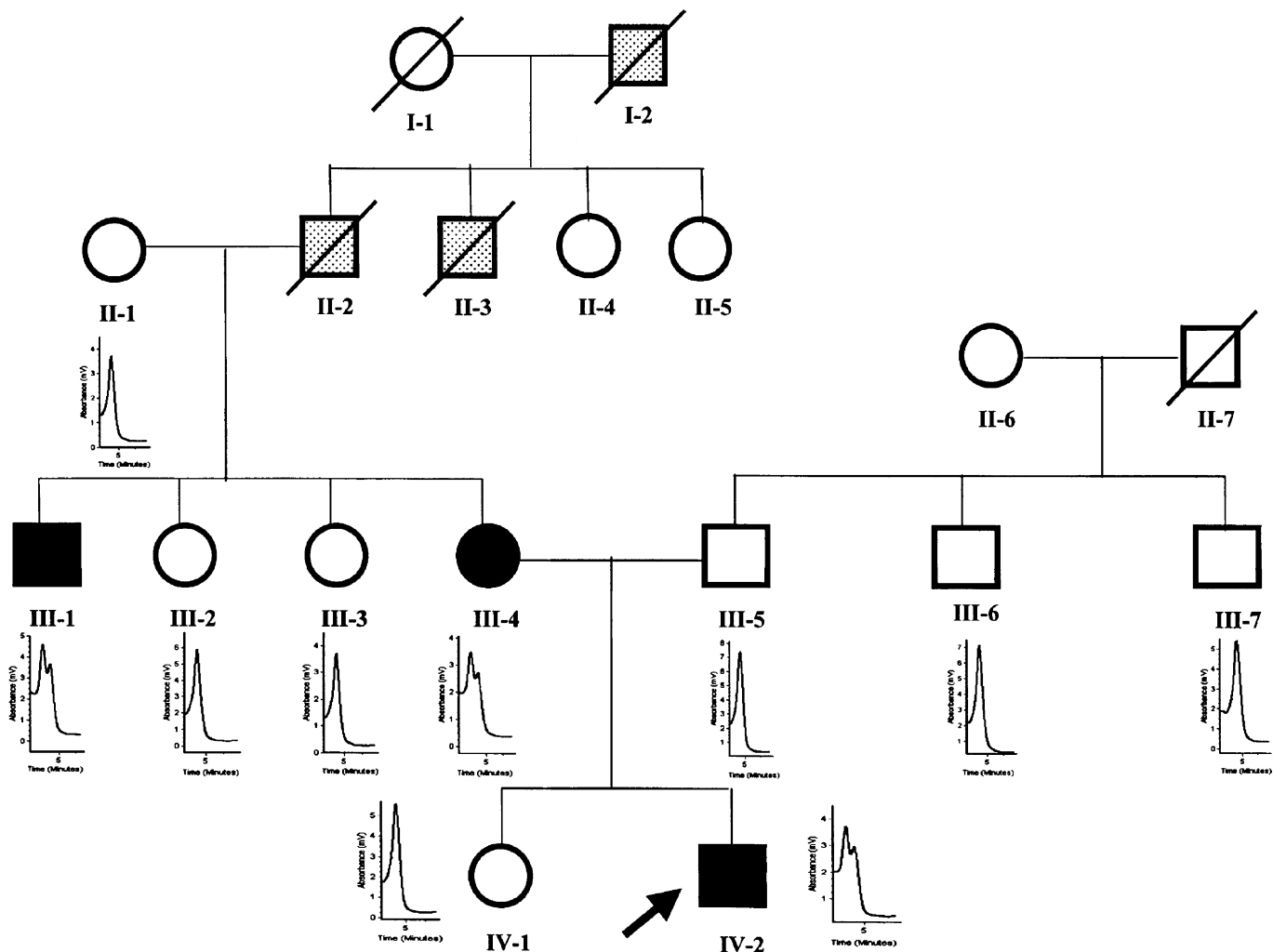
Submitted for publication Dec 11, 2003; last revision received Jun 24, 2004; accepted Aug 3, 2004.

Reprint requests: Elisabetta Cortis, IRCCS Ospedale Pediatrico Bambino Gesù, Piazza S. Onofrio 4, 00165 Roma, Italy. E-mail: [cortis@opbg.net](mailto:cortis@opbg.net). 0022-3476/\$ - see front matter

Copyright © 2004 Elsevier Inc. All rights reserved.

10.1016/j.jpeds.2004.08.001

|        |                                                   |               |                                    |
|--------|---------------------------------------------------|---------------|------------------------------------|
| CD2BP1 | CD2 binding protein 1                             | LPS           | Lipopolysaccharide                 |
| DHPLC  | Denaturing high-performance liquid chromatography | PAPA          | Pyogenic arthritis pyoderma acne   |
| IDDM   | Insulin-dependent diabetes mellitus               | PBMC          | Peripheral blood mononuclear cells |
| JIA    | Juvenile idiopathic arthritis                     | TNF- $\alpha$ | Tumor necrosis factor- $\alpha$    |



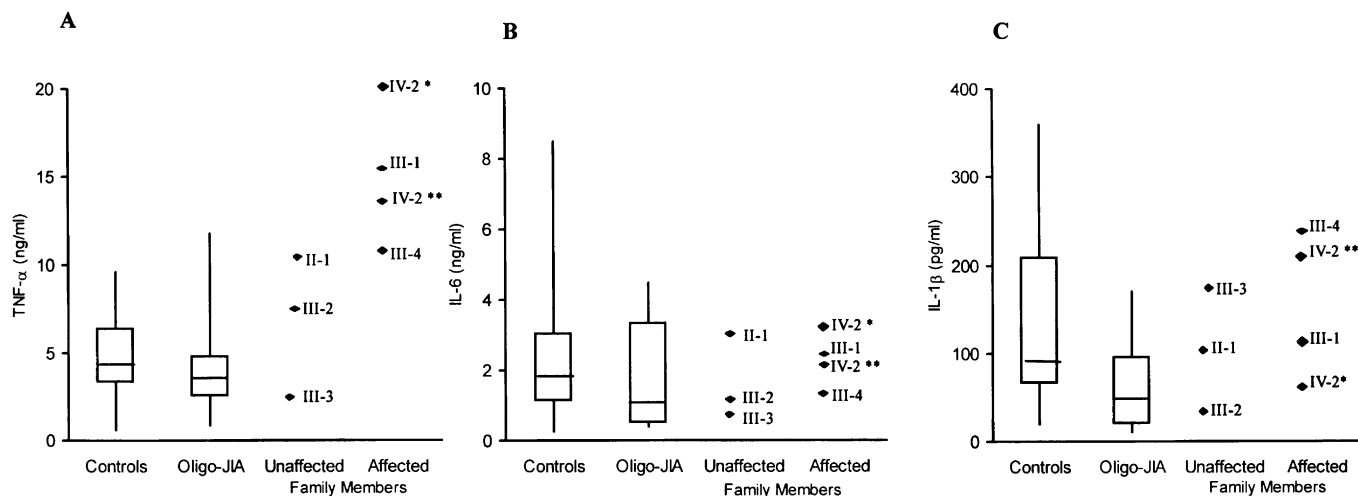
**Figure 1.** Pedigree of the family with PAPA syndrome. DHPLC profiles of exon 10 of CD2BP1 gene are shown for individuals in whom analysis was performed. *Arrow*, proband; *line through symbol*, deceased; *closed symbols*, affected (*III-1*: recurrent arthritis from age 7 years and IDDM at age 35 years; *III-4*: pyoderma gangrenosum at age 12 and IDDM at age 42 years; *IV-2*: see Case Report); *open symbols*, unaffected; *stippled symbols*, history suggestive of PAPA syndrome but unable to confirm (*I-2*: severely crippled by arthritis at young age; *II-2*: some episodes of mild, self-remitting arthritis, died at age 32 years in an accident; *II-3*: recurrent arthritis and diabetes at age 40 years, died at age 45 years).

**Table. Clinical presentation and laboratory investigation of arthritis episodes in the proband (individual IV-2)**

| Age (y) | Affected joint          | Temperature at admission (°C) | ESR (mm/h) | CRP (mg/dL) | WBC count (10 <sup>3</sup> /mm <sup>3</sup> ) | SF leukocyte (×10 <sup>3</sup> /mm <sup>3</sup> ) |     |    |
|---------|-------------------------|-------------------------------|------------|-------------|-----------------------------------------------|---------------------------------------------------|-----|----|
|         |                         |                               |            |             | Neutrophils (%)                               | Neutrophils (%)                                   |     |    |
| 2       | Left shoulder           | 39.0                          | 120        | 18.3        | 28,000                                        | 92                                                | ND  | ND |
| 7       | Left ankle              | 39.2                          | 80         | 18.6        | 11,750                                        | 74                                                | 80  | 92 |
| 8       | Right hip               | 37.8                          | 130        | 21.3        | 12,900                                        | 81                                                | ND  | ND |
| 10      | MCP joints (2°, 3°, 4°) | 39.5                          | 80         | 2.9         | 8900                                          | 61                                                | ND  | ND |
| 16      | Right wrist             | 36.8                          | 14         | 1.5         | 8890                                          | 65                                                | ND  | ND |
| 17      | Right ankle             | 37.8                          | 80         | 2.9         | 7300                                          | 60                                                | 117 | 96 |
| 18      | Right ankle             | 39                            | 50         | 5.8         | 11,300                                        | 68                                                | ND  | ND |
| 19      | Right hip               | 36.8                          | 65         | 3.8         | 4000                                          | 71                                                | 97  | 87 |
| 20      | Right hip               | 37                            | 125        | 7.8         | 9670                                          | 88                                                | ND  | ND |
| 20      | Left knee               | 36.8                          | 54         | 2.6         | 9330                                          | 72                                                | 81  | 90 |
| 20      | Left knee               | 37.6                          | 70         | 5.2         | 10,200                                        | 74                                                | ND  | ND |

ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein; WBC, white blood cell; ND, not determined.





**Figure 2.** Production of proinflammatory cytokines TNF- $\alpha$  (A), IL-6 (B), and IL-1 $\beta$  (C) in healthy control subjects, in patients with oligoarticular JIA (*Oligo-JIA*), and in unaffected and affected individuals of the family with PAPA syndrome. Peripheral blood mononuclear cells were in the presence of 10 ng/mL LPS for 18 hours. For healthy control subjects and patients with *Oligo-JIA*, results are shown as *whisker box*, where limits of boxes represent 25th and the 75th percentile values; *lines across boxes* are median values and *whiskers* depict minimum and maximum values. For unaffected and affected family members, results are shown as individual symbols and are the means of two independent experiments. Two samples of mononuclear cells of individual IV-2 were tested, one obtained before (\*) and the other after (\*\*) etanercept treatment.

of lipopolysaccharide (LPS) (*Escherichia coli*, 055:B5; Sigma, St Louis, Mo) for the indicated time. A neutralizing monoclonal antibody to human interleukin (IL)-1 $\beta$  (R & D Systems) was added at 10  $\mu$ g/mL. The supernatants were harvested, centrifuged, and frozen at  $-80^{\circ}\text{C}$ . IL-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  levels were measured with commercial ELISA (R&D Systems, Minneapolis, Minn, and Biosource Europe, Nivelles, Belgium), according to the manufacturer's instructions.

## CASE REPORT

A 22-year-old man was seen at age 2 years because of left shoulder arthritis, abscess of the left arm, and fever. Erythrocyte sedimentation rate, C-reactive protein, and white blood cell counts were elevated (Table). He was treated with antibiotics and showed slow improvement. His mother had severe necrotic pustules on her face and trunk during adolescence and had development of insulin-dependent diabetes mellitus (IDDM) at 42 years of age. The maternal uncle had severe joint deformities reportedly resulting from relapsing, intensively inflammatory arthritis involving several joints during childhood and had development of IDDM at 35 years.

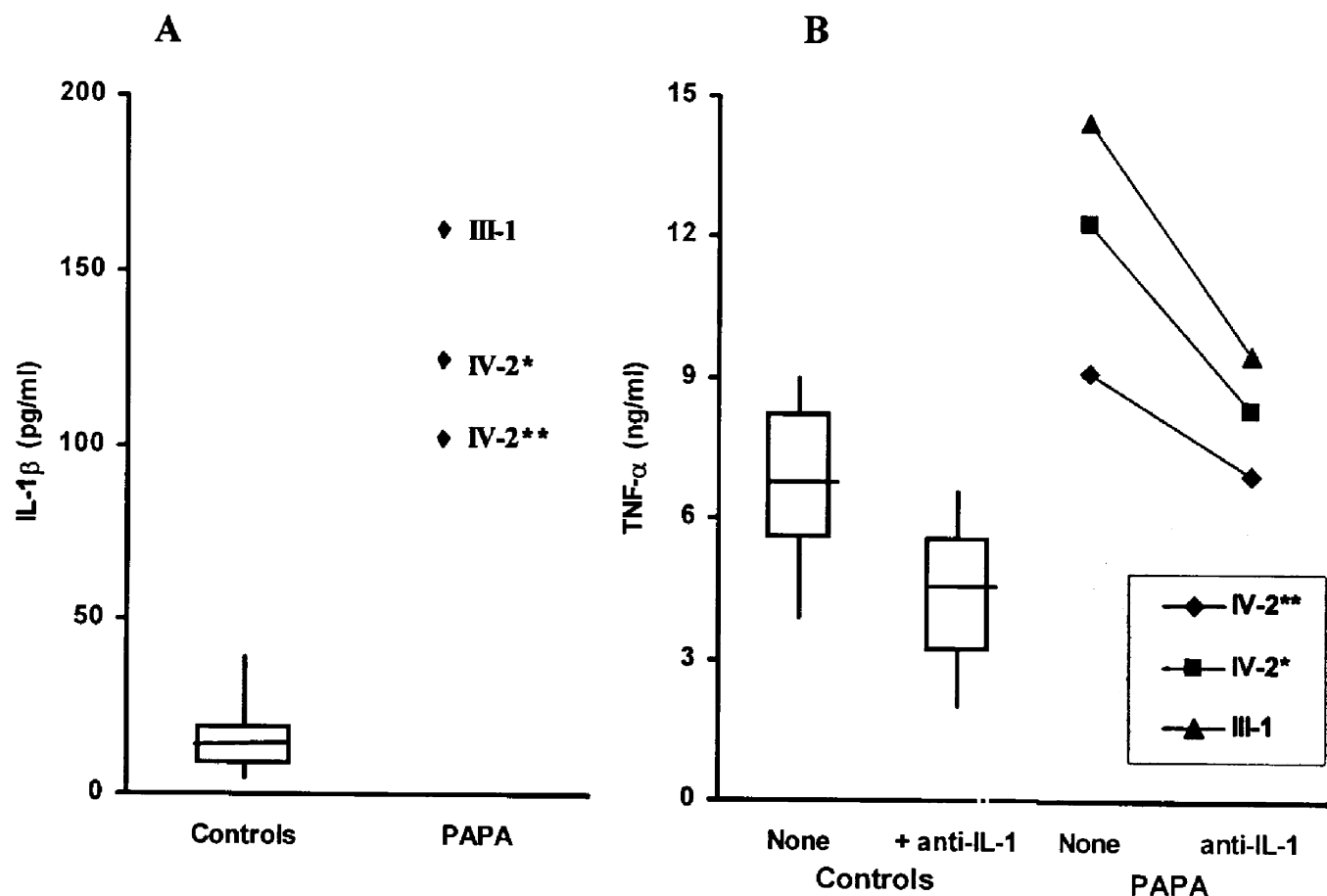
Until 10 years of age, the patient had 3 episodes of arthritis with similar clinical and laboratory features (Table). Synovial fluid was purulent, but cultures were negative. The synovial biopsy showed marked infiltration of neutrophils. The tuberculin test was negative. The NBT test, serum immunoglobulin levels, lymphocyte subsets, ANA, and RF were normal. He was treated with antibiotics and showed slow improvement. He had no additional episodes for 6 years. From the ages of 16 to 19 years, he had 4 similar episodes treated with nonsteroidal anti-inflammatory drugs and showed slow

resolution (Table). PAPA syndrome was diagnosed on clinical data (recurrent pyogenic arthritis with negative synovial cultures) and family history. Prednisone (2 mg/kg per day) was started, resulting in rapid and complete remission. Prednisone was tapered and discontinued in 1 month. Three months later, he had arthritis of the right hip, which was treated with prednisone (2 mg/kg per day). Cyclosporine A (5 mg/kg per day) was added. When prednisone was reduced to 1 mg/kg per day, he had two additional episodes of arthritis of the left knee and a sterile abscess of the quadriceps muscle. Radiography showed narrowing of the joint space and a short and irregular femoral head of the right hip. He also had cystic acne and significant glucocorticoid side effects.

In September 2001, etanercept (25 mg/dose twice weekly), a recombinant soluble TNF receptor II/Fc fusion protein that neutralizes TNF,<sup>5</sup> was started. Glucocorticoids were tapered and discontinued in 2 months. Up to now, the patient has been receiving etanercept for 30 months. No additional episodes of arthritis occurred, and cystic acne resolved. Moderate limitation of motion of the right hip persists.

## CD2BP-1 Mutation Analysis

Denaturing high-performance liquid chromatography analysis of exon 10 amplicons showed heterozygosity in the proband and in two affected family members (Figure 1). Sequencing identified a wild-type and a mutant sequence with a G  $\rightarrow$  A transition at position 904 in exon 10 of CD2BP-1, identical to one of the reported disease-causing mutations.<sup>3</sup> DHPLC analysis did not reveal additional heterozygous sequence variants in both affected and nonaffected family members.



**Figure 3.** Release of IL-1 $\beta$  after short-term stimulation (2 hours) with 10 ng/mL LPS (A) and production of TNF- $\alpha$  after long-term stimulation (18 hours) in the presence or absence of a neutralizing antibody to human IL-1 $\beta$  (B) in healthy control subjects and in individuals with PAPA syndrome. For healthy control subjects, results are shown as *whisker box*, where limits of boxes represent 25th and the 75th percentile values; *lines across boxes* are median values and *whiskers* depict minimum and the maximum values. For individuals with PAPA syndrome, results are shown as individual symbols and are the means of two independent experiments. Two samples of mononuclear cells of individual IV-2 were tested, one obtained before (\*) and the other after (\*\*) etanercept treatment.

## In Vitro Cytokine Production

TNF- $\alpha$  production after an 18-hour LPS stimulation was higher in affected subjects than in healthy control subjects and in patients with oligoarticular JIA. TNF- $\alpha$  production of genetically normal relatives did not differ from that of control subjects (Figure 2, A). In the proband (IV-2), TNF- $\alpha$  production was high before and during etanercept treatment. Individuals III-1 and III-4 and patients with JIA were not receiving glucocorticoids or TNF inhibitors at sampling. No difference was found in IL-6 and IL-1 $\beta$  production after an 18-hour stimulation (Figure 2, B and C).

In agreement with a recent observation,<sup>4</sup> IL-1 $\beta$  release was markedly increased in subjects with PAPA syndrome after a short-term (2-hour) stimulation (Figure 2, A). To assess whether the early increase in IL-1 $\beta$  production could be responsible for the late increase in TNF- $\alpha$  production, we evaluated TNF- $\alpha$  production after an 18-hour stimulation with LPS in the presence of a neutralizing antibody to IL-1 $\beta$ . Neutralization of IL-1 $\beta$  resulted in reduction of TNF- $\alpha$

production in control subjects and in patients. However, in the patients, TNF- $\alpha$  production was still markedly higher than that of control subjects (Figure 3, B), suggesting that the increased production of TNF- $\alpha$  after a long incubation (18 hours) was partially secondary to the early IL-1 $\beta$  release.

## DISCUSSION

The study of a subject with PAPA syndrome, of his affected mother and maternal uncle—the third family reported in the literature—showed that PBMC of the affected subjects produce markedly increased amounts of TNF- $\alpha$ . Treatment of the proband with the TNF inhibitor etanercept resulted in clinical long-lasting remission.

In addition to the proband, 2 family members had genetically confirmed PAPA syndrome and 3 were reported by history to have presented features compatible with PAPA syndrome. In 4 of 5, arthritis was prominent, consistent with the observation that recurrent, intensively inflammatory

arthritis is a main feature of the disease. In addition to skin lesions, 3 family members had IDDM with onset in adulthood. Interestingly, adult-onset IDDM has also been described in 1 of the 2 reported families.<sup>2</sup> In our family, antibodies to insulin were present in the mother (37.2 arbitrary units [AU], normal <3) and in the maternal uncle (280 AU). In addition, the mother had high antibody titers to glutamic acid decarboxylase (>100 AU, normal <3). The proband is presently negative for autoantibodies to islet antigens. All three affected family members carry an HLADR3 allele. These findings, together with the previous observation of IDDM in one of the reported families, suggest that adult-onset autoimmune diabetes may represent a feature of PAPA syndrome.

After an initial response, our patient had resistance to glucocorticoids with recurrences on a daily regimen, therefore showing that failure to respond satisfactorily to glucocorticoids may occur in PAPA syndrome. The observation that administration of the TNF inhibitor etanercept was associated with a rapid, sustained clinical remission suggests that etanercept represents a valuable therapeutic option in patients with unsatisfactory response to glucocorticoids. It also underscores the possible role of TNF- $\alpha$  in the inflammatory process of this disease. The high in vitro TNF- $\alpha$  production of patients with genetically confirmed PAPA syndrome supports this hypothesis.

The recent observation that PAPA-associated CD2BP1 mutants bind pyrin with high affinity,<sup>4</sup> together with our findings, suggest that mutation in CD2BP1 leads to deranged regulation of the inflammatory response. Pyrin binds to and inhibits ASC, a protein involved in recruitment and activation of caspase-1, which in turn is responsible for processing pro-IL-1 $\beta$  in its mature form.<sup>6</sup> Pyrin-deficient mice show increased IL-1 $\beta$  processing with increased mature IL-1 $\beta$  release.<sup>7</sup> On the basis of these observations, it was hypothesized that sequestering of pyrin, caused by increased pyrin binding by CD2BP1 mutants, could lead to increased IL-1 $\beta$  processing. Indeed, Shoham et al<sup>4</sup> observed that one patient with PAPA syndrome showed markedly increased IL-1 $\beta$  release after a short-term stimulation with LPS. We have confirmed this observation showing in 2 additional patients with PAPA syndrome that a short stimulation leads to a marked increase in IL-1 $\beta$  release. In addition, we have shown that PAPA-associated CD2BP1 mutation does not directly affect in vitro IL-1 $\beta$  production after a long-term stimulation (18 hours), involving gene expression, mRNA

translation, and protein processing. On the other hand, we have shown that individuals with PAPA syndrome produce high amounts of TNF- $\alpha$  after an 18-hour stimulation and that this increased production can only partially be explained by the early release of IL-1 $\beta$ . The latter findings suggest that CD2BP1 mutations may affect inflammatory cytokine production through different pathways. These may again involve the interactions with pyrin and ASC. Indeed, ASC has been shown not only to mediate caspase-1 recruitment and activation but also to modulate Nuclear Factor- $\kappa$ B signaling pathways, suggesting broader modulatory effects on inflammatory responses.<sup>8</sup> High production of IL-6, TNF- $\alpha$ , and IL-8 has been reported in attack-free patients with familial mediterranean fever, supporting the hypothesis for a role of pyrin and ASC in the modulation of several inflammatory cytokines, in addition to IL-1 $\beta$ .<sup>9</sup>

## REFERENCES

1. Jacobs JC, Goetzl EJ. Streaking leukocyte factor, arthritis, and pyoderma gangrenosum. *Pediatrics* 1975;56:570-8.
2. Lindor NM, Arsenault TM, Solomon H, Seidman CE, McEvoy MT. A new autosomal dominant disorder of pyogenic sterile arthritis, pyoderma gangrenosum and acne: PAPA syndrome. *Mayo Clin Proc* 1997;72:611-5.
3. Wise CA, Giliem JD, Seidman CE, Lindor NM, Veile R, Bashardes S, et al. Mutations in CD2BP1 disrupt binding to PTP PEST and are responsible for PAPA syndrome, an autoinflammatory disorder. *Hum Mol Genet* 2002;11:961-9.
4. Shoham NG, Centola M, Mansfield E, Hull KM, Wood G, Wise CA, Kastner DL. Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial mediterranean fever and PAPA syndrome as disorders in the same pathway. *Proc Natl Aca Sci U S A* 2003;100:13501-6.
5. Lovell DJ, Giannini EH, Reiff A, Cawkwell GD, Silverman ED, Necton JJ, et al. Etanercept in children with polyarticular juvenile rheumatoid arthritis. *N Engl J Med* 2000;342:763-9.
6. Martinon F, Burn K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-1 $\beta$ . *Mol Cell* 2002;10:417-26.
7. Chae JJ, Komarow HD, Cheng J, Wood J, Raben N, Liu PP, Kastner DL. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol Cell* 2003;11:591-604.
8. Stehlik C, Fiorentino L, Dorfleitner A, Bruey JM, Ariza EM, Reed JC. The PAA/PYRIN-Family protein ASC is a dual regulator of a conserved step in Nuclear Factor  $\kappa$ B activation pathways. *J Exp Med* 2002;196:1605-15.
9. Notaricola C, Didelot MN, Seguret F, Demaille J, Toutou I. Enhanced cytokine mRNA levels in attack-free patients with familial mediterranean fever. *Genes Immunol* 2002;3:43-5.

# Mechanistic Associations of a Mild Phenotype of Immunodysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome

FABRIZIO DE BENEDETTI,\* ANTONELLA INSALACO,<sup>†</sup> ANTONELLA DIAMANTI,<sup>§</sup> ELISABETTA CORTIS,<sup>†</sup> FLAMINIA MURATORI,\* ANDREA LAMIONI,\* RITA CARSETTI,\* ROBERTO CUSANO,<sup>¶</sup> RITA DE VITO,<sup>¶</sup> LUCIA PERRONI,<sup>¶</sup> MANUELA GAMBARARA,<sup>§</sup> MASSIMO CASTRO,<sup>§</sup> GIAN FRANCO BOTTAZZO,\* and ALBERTO G. UGAZIO<sup>†</sup>

\*Direzione Scientifica, <sup>†</sup>Pediatria II–Reumatologia, <sup>§</sup>Gastroenterologia, and <sup>¶</sup>Anatomia Patologica, Istituto di Ricovero e Cura a Carattere Scientifico Ospedale Pediatrico Bambino Gesù, Roma, Italy; and the <sup>¶</sup>Laboratorio Genetica Umana, Ospedali Galliera, Genova, Italy

**Background & Aims:** The syndrome of immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is a rare disorder resulting in the expression of multiple autoimmune and allergic features. Early onset enteropathy and type 1 diabetes (T1D) are the most common clinical features. The IPEX syndrome is caused by mutations of the FOXP3 gene, which is essential for the development of regulatory T cells (Treg). We describe 2 unrelated patients with IPEX syndrome with a mild clinical phenotype and with novel FOXP3 mutations and the phenotypic and functional characterization of their Treg cells. **Methods:** The FOXP3 gene was analyzed by sequencing amplicons from genomic DNA. Treg cells were characterized by evaluating the number of CD4<sup>+</sup>CD25<sup>+</sup> T cells and their functional ability to suppress the proliferation of autologous CD4<sup>+</sup>CD25<sup>–</sup> effector T cells stimulated with anti-CD3 and anti-CD28 antibodies. **Results:** A 7-year-old boy and a 24-year-old man presented with autoimmune enteropathy characterized by early onset persistent diarrhea not associated with T1D or other endocrinopathies. These 2 patients carry novel FOXP3 mutations that do not abrogate the function of the forkhead domain. They have normal numbers of CD4<sup>+</sup>CD25<sup>+</sup> T lymphocytes, however, these show severely defective suppressive function in vitro. **Conclusions:** Our 2 patients show that IPEX patients may present with early onset enteropathy and long-term survival without T1D or other endocrinopathies. This milder phenotype may be associated with FOXP3 mutations that do not abrogate the function of the forkhead domain.

The syndrome of immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is characterized by multiple autoimmune and allergic features.<sup>1–3</sup> Early onset enteropathy, with villous atrophy and infiltrating inflammatory cells, is the most common manifestation. It is difficult to treat and is the primary cause

of death. Early onset type 1 diabetes (T1D), with autoantibodies to islet antigens, usually is present and other autoimmune endocrinopathies are common, together with thrombocytopenia, hemolytic anemia, and arthritis.<sup>1,4</sup> Asthma and eczema, with increased serum immunoglobulin (Ig)E levels, are frequent. Most reported patients with IPEX have died in infancy or early childhood.

IPEX is caused by mutations of the FOXP3 gene encoding a transcription factor, named *scurfin*, of the forkhead family.<sup>5</sup> Scurfin is a DNA-binding protein that suppresses cytokine gene expression in activated T lymphocytes, possibly through direct repression of NFAT.<sup>6</sup> In human beings, 13 FOXP3 mutations have been reported.<sup>4</sup> Ten disrupt the forkhead domain, 2 affect the leucine zipper domain, and 1 affects the first polyadenylation signal. The forkhead domain is required, both in humans and mice, for FOXP3 function.<sup>6</sup> The impact of mutations in the leucine zipper domain and in the polyadenylation site on expression and function of FOXP3 still is unknown.

FOXP3-deficient mice have a disease similar to IPEX.<sup>7</sup> In mice, FOXP3 is essential for the development of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) and transplantation of Tregs from a normal strain rescues the clinical phenotype.<sup>8</sup> Human Treg cells, also included in the CD4<sup>+</sup>CD25<sup>+</sup> population, inhibit proliferation and cytokine production of effector T cells and have been implicated in several immune-mediated disorders.<sup>9</sup> We report

**Abbreviations used in this paper:** ANA, antinuclear antibody; bp, base pair; cDNA, complementary DNA; ESR, erythrocyte sedimentation rate; Ig, immunoglobulin; IPEX, immunodysregulation, polyendocrinopathy, enteropathy, X-linked; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; T1D, type 1 diabetes; Treg, regulatory T cells.

© 2006 by the American Gastroenterological Association Institute  
1542-3565/06/\$32.00  
doi:10.1016/j.cgh.2005.12.014



2 unrelated patients with IPEX and a mild clinical phenotype characterized by early onset enteropathy, absence of T1D or other endocrinopathies, and prolonged survival. These patients carry novel FOXP3 mutations that do not disrupt the forkhead domain and show a functional defect of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells.

## Case Reports

### Patient 1

A 22-year-old man was referred because of recurrent diffuse joint pain associated with the presence of antinuclear antibodies (ANAs). The mother died at 42 years of age of intestinal lymphoma. The patient had 3 brothers. One brother died at 1 month of age from intractable diarrhea; diabetes was not reported. The other 2 brothers, both asymptomatic, had biopsy examination–proven celiac disease and responded to a gluten-free diet. One brother died at 14 years of age in an accident; the other brother, aged 26 years, was lost to follow-up evaluation.

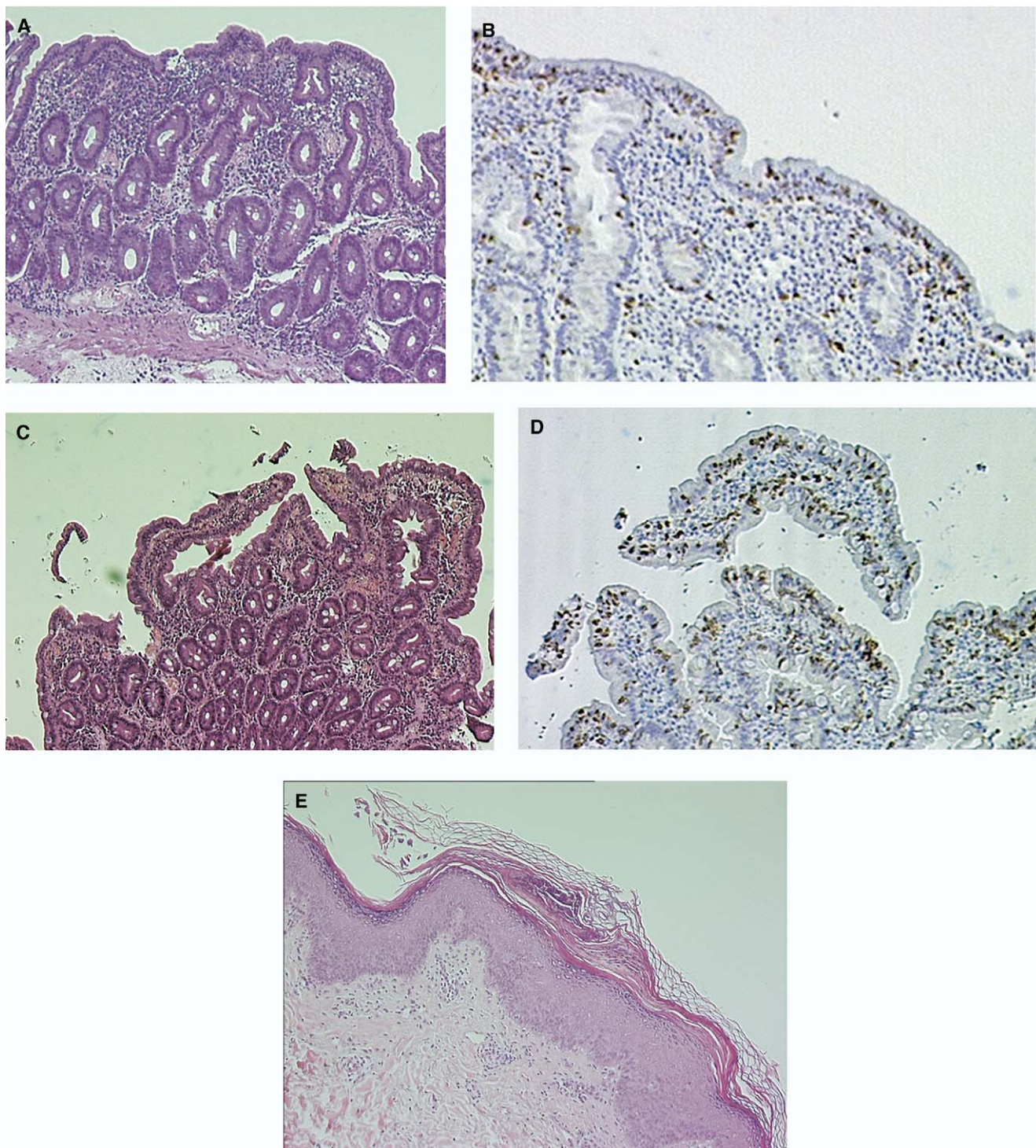
At 18 days of age, patient 1 developed diarrhea and severe malnutrition. Parenteral nutrition and a subsequent diet with a peptide formula reportedly were associated with improvement and he was maintained on a gluten-free diet. At 5 years, after reintroduction of a full gluten-containing diet, abdominal pain, diarrhea, and failure to thrive relapsed. An intestinal biopsy examination showed subtotal villous atrophy and mononuclear cell infiltrate in the lamina propria (Figure 1A). Immunohistochemistry subsequently was performed and revealed an intraepithelial CD3<sup>+</sup>CD8<sup>+</sup> cell count of 42/100 epithelial cells (upper limit of normal, 40) (Figure 1B). High titers of antigliadin IgG and antiendomysial antibodies were found. Celiac disease was diagnosed. After an initial clinical improvement with a gluten-free diet, diarrhea rapidly relapsed and failure to thrive persisted. Two years later a second intestinal biopsy examination showed similar findings (not shown). IgE levels were high (200 mU/mL; normal values for age, <60 mU/mL), reportedly in the absence of allergy. Diarrhea and failure to thrive persisted. At 15 years, the patient developed arthralgia and transient arthritis. Antienterocyte antibodies were detected for the first time in this patient (immunofluorescence on human duodenum; Dr. E. Bosi, San Raffaele Research Institute, Milan, Italy). A diagnosis of autoimmune enteropathy was made and treatment with glucocorticoids and azathioprine was started with partial improvement. At 18 years the patient developed well-defined, scaly, erythematous plaques on the trunk and limbs. A skin biopsy examination showed parakeratosis, Poitrier-like epidermal

microabscesses, and mononuclear cell infiltrate in perivascular aggregates, consistent with psoriasiform dermatitis (Figure 1E).

Chronic enteropathy remained active until admission to our unit despite continuous treatment with glucocorticoids, immunosuppressants (cyclosporin A, tacrolimus), and infliximab, alone or in combination, and intermittent parenteral nutrition. At admission, the patient was receiving prednisone, cyclosporin A, and nightly parenteral nutrition. His height was 164 cm (25th–50th percentile), and his weight was 48 kg (10th–25th percentile). He was in fair general condition, presenting with weakness and mild signs of dehydration. Maculopapular lesions were present over the limbs and trunk. The liver was mildly enlarged. The white blood cell count was 10,570/mm<sup>3</sup>, hemoglobin level was 12.1 g/dL, platelet count was 219,000/mm<sup>3</sup>, erythrocyte sedimentation rate (ESR) was 28 mm/h (normal, <20 mm/h), and the C-reactive protein level was 39 mg/L (normal, <5 mg/L). Metabolic acidosis (pH 7.30; bicarbonate, 13.3 mEq/L) and renal failure (serum creatinine level, 2.59 mg/dL; urea nitrogen level, 42 mg/dL) were present. Blood electrolyte, glucose, and liver enzyme levels were normal. ANAs were positive (1:160; homogeneous pattern). Antitransglutaminase antibodies were negative. The patient was treated with 3 intravenous methylprednisolone pulses (1 g/day) and parenteral rehydration. He was discharged 1 week later in good general condition with prednisone (1 mg/kg/day in 1 daily dose), tacrolimus (.04 mg/kg/day), azathioprine (2 mg/kg/day), and nightly parenteral nutrition. His weight was 50 kg, creatinine level was 1.45 mg/dL, and bicarbonate level was 22.0 mEq/L.

### Patient 2

A 5-year-old boy was referred to our unit because of pain in the wrist and the left proximal interphalangeal joint of the second finger and diffuse arthralgia. His 12-year-old brother was healthy. Severe chronic diarrhea had appeared at 14 months of age. An intestinal biopsy examination showed dysmorphic villi with no atrophy and a marked mononuclear cell infiltrate in the lamina propria (Figure 1C) and an intraepithelial CD3<sup>+</sup>CD8<sup>+</sup> cell count of 60/100 epithelial cells (upper limit of normal, 40) (Figure 1D). Antigliadin and antiendomysial antibodies were negative. The patient did not improve on a gluten-free diet. IgE levels were 74 mU/mL (normal value for age, <10 mU/mL). Antienterocyte antibodies were negative. ANAs were present (1:5120, homogenous pattern). The patient was diagnosed with an unspecified autoimmune enteropathy and treatment with prednisone, azathioprine, and exclusive intravenous nutrition



**Figure 1.** Intestinal biopsy specimen of (A, B) patient 1 and (C, D) patient 2, and a skin biopsy specimen of (E) patient 1. (B, D) Immunohistochemistry of the intestinal biopsy specimen with an anti-CD3 antibody.

was started. Substantial clinical improvement and weight gain ensued. At 18 months of age the patient developed pneumonia with pleuritis, and pericarditis a month later. He was treated with prednisone and azathioprine; treatment was tapered and eventually with-

drawn at 3 years and 5 months. Since then he had been growing satisfactorily on a full gluten-containing diet.

At admission, he was in good clinical condition. His height was 110 cm (50th–75th percentile) and his weight was 16 kg (10th–25th percentile). Physical ex-



**Table 1.** CD4<sup>+</sup>CD25<sup>+</sup> T Cells in Patients With IPEX Syndrome, Healthy Controls, and in Patients With Autoimmune Diseases Treated With Glucocorticoids and Immunosuppressants

|                          | CD25 <sup>+</sup> total (% of CD4 <sup>+</sup> ) | CD25 <sup>+</sup> bright (% of CD4 <sup>+</sup> ) | CD69 <sup>+</sup> (% of CD4 <sup>+</sup> CD25 <sup>+</sup> ) |
|--------------------------|--------------------------------------------------|---------------------------------------------------|--------------------------------------------------------------|
| Healthy controls (range) | 6.5–10.7                                         | .48–1.72                                          | .6–4.2                                                       |
| IPEX (patient 1)         | 7.3                                              | .91                                               | 1.95                                                         |
| IPEX (patient 2)         | 6.6                                              | 1.01                                              | 1.79                                                         |
| Autoimmune/treatment     |                                                  |                                                   |                                                              |
| Control 1                | 8.53                                             | .62                                               | 2.41                                                         |
| Control 2                | 9.2                                              | 1.99                                              | 1.89                                                         |
| Control 3                | 6.92                                             | 1.05                                              | 1.29                                                         |
| Control 4                | 10.38                                            | 2.40                                              | .51                                                          |

Autoimmune/treatment: control 1, systemic juvenile idiopathic arthritis treated with prednisone (.4 mg/kg/day) and cyclosporin A (4 mg/kg/day); control 2, juvenile dermatomyositis treated with cyclosporin A (5 mg/kg/day); control 3, systemic juvenile idiopathic arthritis treated with prednisone (.1 mg/kg/day) and cyclosporin A (5 mg/kg/day); control 4, systemic lupus erythematosus treated with prednisone (.4 mg/kg/day) and azathioprine (2 mg/kg/day).

amination showed moderate swelling of the wrists and of the left proximal interphalangeal joint of the second finger. No other signs or symptoms were present. Blood cell counts and liver and kidney functions were normal. ESR was 34 mm/h, the C-reactive protein level was 3.3 mg/L. ANAs were confirmed at a titer of 1:5120 with a homogenous pattern. He was treated with naproxen (15 mg/kg/day). In the subsequent weeks, arthritis and arthralgia persisted and he presented with occasionally watery diarrhea. Treatment with prednisone and azathioprine was started and was followed by a rapid resolution of arthritis and diarrhea. After 2 years, the patient is presently a 7-year-old boy in good health, regularly attending school, who has not presented with recurrences of his enteropathy, and is being treated with azathioprine (2 mg/kg/day) and prednisone (presently .2 mg/kg/day on an alternate-day regimen). ANAs still are present (1:2560). The IgE level is 7.6 IU/mL (normal values for age, <100 IU/mL).

### Additional Information

The following investigations were normal or negative in both patients: oral glucose tolerance test, hemoglobin-A1c, fructosamine, insulin-like growth factor, dehydroepiandrosterone-S, thyroid functions, including a thyroid ultrasound, antibodies to glutamic acid decarboxylase, thyroid (thyroglobulin and thyroid-peroxidase), smooth muscle, liver-kidney microsomes, mitochondria, extractable nuclear antigens, cardiolipin, and neutrophil cytoplasm.

## Materials and Methods

### Mutation Analysis

The 11 exons, including the intron-exon junctions, of the FOXP3 gene were amplified from genomic DNA by polymerase chain reaction (PCR), sequenced by the Big Dye

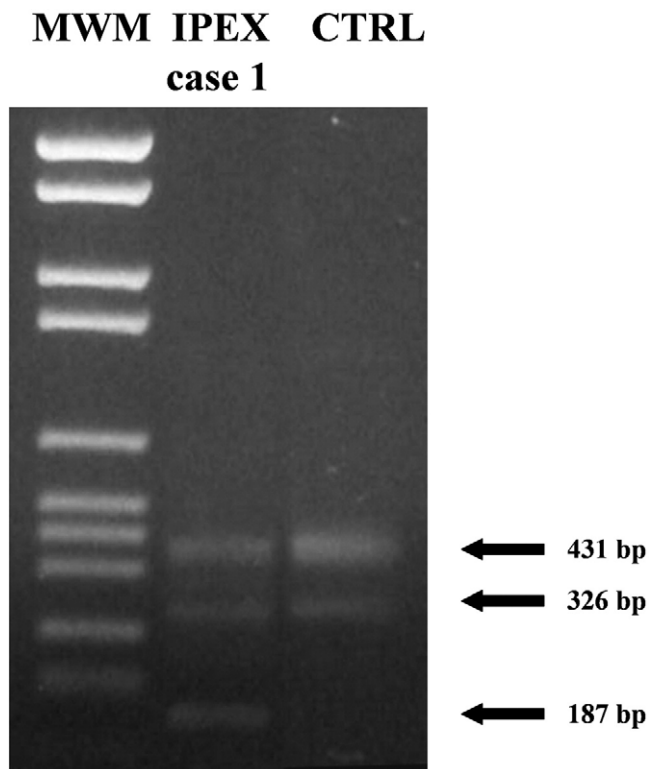
Terminator kit version 1.1 (Applied Biosystems, Warrington, Cheshire, UK) and the sequences were run on an ABI Prism 377 (Applied Biosystems). A panel of 200 control men was analyzed by denaturing high pressure liquid chromatography to exclude that the nucleotide variations detected in the 2 patients were polymorphisms.

For the expression analysis in patient 1, the total RNA was obtained from peripheral blood mononuclear cells (PBMCs) using TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription was performed with the Advantage reverse transcription for PCR kit (Clontech, Palo Alto, CA). Complementary DNA (cDNA) spanning from exons 1 to 5 was amplified by PCR, with specific primers. Transcripts were cloned by TOPO TA Cloning kit (Invitrogen, Life Technologies Inc.) and selected colonies, representing all the PCR products observed, were sequenced.

### Evaluation of the Phenotype and Function of Regulatory T Cells

At the time of sampling, patient 1 was receiving prednisone (.5 mg/kg/day) and cyclosporin A (7 mg/kg/day), and patient 2 was receiving prednisone (.2 mg/kg/day) and azathioprine (2 mg/kg/day). Two independent blood samples from the 2 patients were analyzed. Samples from 4 patients with autoimmune diseases treated with the same immunosuppressants (Table 1) were used as disease/treatment controls. Ten healthy young adults (age, 22–40 y) served as normal controls.

PBMCs were prepared by centrifugation on Ficoll-Hypopaque (Sigma Chemicals, St. Louis, MO). Monoclonal antibodies for flow cytometry and sorting were from Becton-Dickinson (San Jose, CA). For flow cytometry,  $5 \times 10^5$  PBMCs were stained with appropriate antibody combinations of fluorescein, phycoerythrin, allophycocyanin, and cytochrome-conjugated monoclonal antibodies; in particular, FN50 (anti-CD69), M-A251 (anti-CD25), RPA-T4 (anti-CD4), UCHT1 (anti-CD3), G42-8 (anti-CD8), HIB19 (anti-CD19), and 3G8 (anti-CD16). Cells were analyzed by FACSCalibur (Becton-Dickinson). For sorting, PBMCs were stained with fluorescein



**Figure 2.** FOXP3 transcripts in patient 1 and in 1 healthy control. The 431-bp fragment corresponds to the full-length cDNA produced by healthy patients; the 326-bp transcript found in the patient and in some of the normal controls represents an alternative splicing with skipping of exon 2 and conserved frame. The 187-bp transcript skips exons 2 and 3 and results in an altered reading frame with a premature stop.

isothiocyanate-conjugated anti-CD8, CD16, and CD19 antibodies, and phycoerythrin-conjugated anti-CD25 antibodies. CD4<sup>+</sup> T cells were identified as CD8, CD16, CD19, and CD25 negative. The CD4<sup>+</sup>CD25<sup>+</sup> T cells, containing Treg cells, were negative for all markers, except for CD25. The cell populations were separated using the FACSVantage SE (Becton-Dickinson). Sorted T cells (CD4<sup>+</sup> or Treg-depleted CD4<sup>+</sup>) were cultured as described previously<sup>10</sup> for 4 days in triplicate in 96-well plates in the presence of irradiated (3000 rads) autologous PBMCs in serum-free X-VIVO-15 medium (Bio-Whittaker Inc, Walkersville, MD) and stimulated with 2  $\mu$ g/mL of soluble anti-CD3 and .15  $\mu$ g/mL of soluble anti-CD28 (Becton-Dickinson). During the final 16 hours, .5  $\mu$ Ci/well of <sup>3</sup>H-thymidine was added and proliferation was evaluated by thymidine uptake.

## Results

### Mutation Analysis

Patient 1 carries an A→G transition at +4 in intron 3 (454+4A>G), very close to the splice site. Reverse-transcription PCR was performed on RNA from PBMCs to obtain cDNA corresponding to exons 1–5.

Three cDNA transcripts of 431, 326, and 187 base pairs (bp) were detected (Figure 2). Cloning and sequencing of the 3 fragments showed that the 431-bp fragment corresponds to the full-length cDNA produced by healthy subjects; the 326-bp transcript also is found in some normal controls and represents an alternative splicing with skipping of exon 2 and conserved frame (GenBank AJ005891); in the 187-bp transcript skipping of exons 2 and 3 was seen, resulting in a frame shift. The putative resulting protein has an abnormal amino-acid composition and it is truncated owing to a premature stop codon in exon 7.

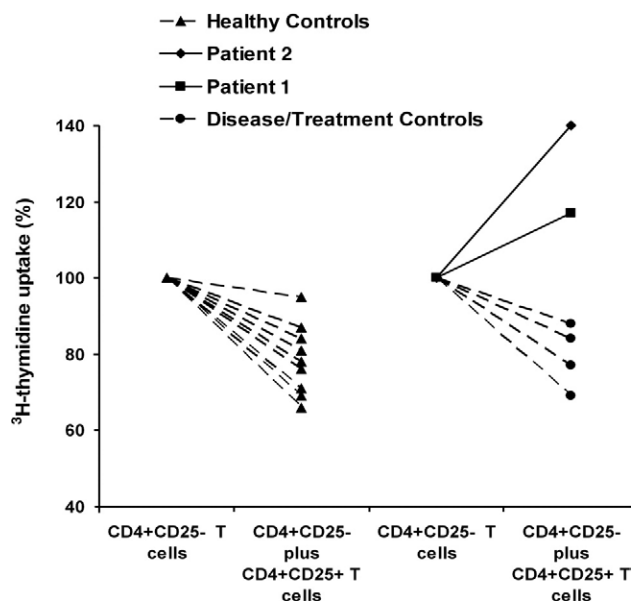
Patient 2 is hemizygous for a C→T transition in exon 3 at nt323, causing the substitution of a threonine with methionine at position 108 (T108M). This missense mutation affects the proline-rich domain and the resulting protein has a predicted normal forkhead domain. The mutations of the 2 patients were not found in the screening of the 200 healthy adult men.

### Phenotype and Function of Regulatory T Cells

The 2 IPEX patients had a CD4<sup>+</sup> T-cell subpopulation that constitutively expressed CD25. Flow cytometry analysis showed that the percentage of CD4<sup>+</sup>CD25<sup>+</sup> was normal compared with that found in healthy controls (Table 1). Children with other chronic autoimmune diseases who were treated with the same immunosuppressants had similarly normal percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells (Table 1). Treg cells are enriched in the population, expressing high levels of CD25 (CD4<sup>+</sup>CD25<sup>+</sup> T cells). Evaluation of CD4<sup>+</sup>CD25<sup>+</sup> T cells showed no differences from healthy controls (Table 1). In both IPEX patients, similar to disease/treatment control patients, a negligible percentage of the CD4<sup>+</sup>CD25<sup>+</sup> T cells expressed the activation marker CD69 (Table 1), therefore suggesting that antigen-activated T cells did not contribute significantly to the subset of CD4<sup>+</sup>CD25<sup>+</sup> T cells.

To investigate the function of the CD4<sup>+</sup>CD25<sup>+</sup> population, we evaluated the ability of CD4<sup>+</sup>CD25<sup>+</sup> to suppress the proliferation of autologous CD4<sup>+</sup>CD25<sup>−</sup> T cells. CD4<sup>+</sup>CD25<sup>+</sup> T cells from healthy controls suppressed the proliferation of autologous effector T cells by 15%–40% (Figure 3). On the contrary, coculture of CD4<sup>+</sup>CD25<sup>+</sup> T cells from IPEX patients with autologous T cells did not result in any suppression, but rather in increased proliferation (Figure 3). CD4<sup>+</sup>CD25<sup>+</sup> T cells from disease/treatment controls suppressed proliferation of autologous T cells in a range similar to that of healthy controls, excluding that the lack of suppression





**Figure 3.** Proliferation of effector CD4<sup>+</sup>CD25<sup>-</sup> T lymphocytes in the absence or in the presence of CD4<sup>+</sup>CD25<sup>+</sup> Tregs from healthy controls, from autoimmune/treatment controls, and from the 2 IPEX patients. CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> populations were purified by sorting and were stimulated with anti-CD3 and anti-CD28 as described in the Materials and Methods section. Results are expressed as a percent of thymidine uptake of stimulated effector CD4<sup>+</sup>CD25<sup>-</sup> T lymphocytes cultured in the absence of CD4<sup>+</sup>CD25<sup>+</sup> T cells. ▲, Healthy controls; ◆, patient 2; ■, patient 1; ●, disease/treatment controls.

by the CD4<sup>+</sup>CD25<sup>+</sup> cells of IPEX patients was secondary to treatment.

## Discussion

The course of our patients appears to be milder than that of other IPEX patients reported. Both of our patients survived the early years of life and patient 1 already has reached young adulthood. In both of our patients early onset severe enteropathy was the prominent clinical feature. In addition, our patients did not develop clinical or subclinical (presence of autoantibodies) evidence of T1D or other autoimmune endocrinopathies, or severe or unusual infections despite chronic immunosuppression.

In patient 1 the intestinal biopsy examination showed subtotal villous atrophy, an intraepithelial lymphocyte count greater than 40%, and mononuclear cell infiltrate. This was classified as stage 3a, according to the modified Marsh classification,<sup>11</sup> and the persistently high titers of antigliadin and antiendomysial antibodies were consistent with a diagnosis of celiac disease. However, patient 1 did not respond to a gluten-free diet. Whether he is affected with both IPEX and celiac disease, or whether the features compatible with celiac disease are manifes-

tations of the immune dysregulation of IPEX, is difficult to ascertain. From a practical point of view, this case indicates that in patients with early onset enteropathy and celiac disease-related antibodies, a failure to respond to a gluten-free diet should prompt the suspicion of IPEX syndrome, even in the presence of histologic findings highly suggestive of celiac disease.

To date, the majority of FOXP3 mutations reported in IPEX patients are either missense mutations in the forkhead DNA binding domain, or deletions resulting in an altered, truncated, or missing forkhead domain.<sup>1,4</sup> In patient 2, a missense mutation in exon 3 leads to an amino-acid substitution that does not affect the forkhead domain. In patient 1, the mutation close to the splice site generates abnormal and normal transcripts, suggesting that at least a certain amount of normal FOXP3 protein is produced. This phenomenon (ie, splicing mutations resulting in abnormal and some normal transcripts), not reported in IPEX, already has been described in X-linked adrenoleukodystrophy.<sup>12</sup> Therefore it is tempting to speculate that a milder IPEX phenotype may be associated with FOXP3 mutations that do not abrogate the function of the forkhead domain. Further studies of genotype/phenotype correlation are needed. Interestingly, the only FOXP3 mutation associated with a mild IPEX phenotype reported to date is that of the family described by Powell et al,<sup>2</sup> which affected the first polyadenylation signal, possibly resulting in messenger RNA destabilization and in reduced production of a normal protein.<sup>13</sup>

In mice, FOXP3 has been shown to be essential for the development and function of Treg cells. No data are available on the function of Treg cells in the patients with IPEX. The percentage and absolute number of circulating CD4<sup>+</sup>CD25<sup>+</sup> T cells were in the low-normal range in both our patients. Because only a negligible percentage of these cells expressed the activation marker CD69, these CD4<sup>+</sup>CD25<sup>+</sup> T cells are unlikely to represent activated T cells. The number of CD4<sup>+</sup>CD25<sup>+</sup> has been reported only in 1 patient with IPEX.<sup>2,14</sup> In this case CD4<sup>+</sup>CD25<sup>+</sup> were found to be within the normal range; however, increased expression of activation markers suggested that they were antigen-activated T cells.<sup>14</sup> Methodologic difference (activation markers used) or difference in the age of the patients (ie, there was only 1 patient in Bakke et al<sup>14</sup> and he was followed-up until age 18 months) could account for this apparent discrepancy. We found that the CD4<sup>+</sup>CD25<sup>+</sup> T cells of both our patients were unable to perform a typical function of Treg cells in vitro: suppression of proliferation of activated T cells. Because anergy (absence of response to

T-cell receptor cross-linking) is another feature of cultured Treg cells,<sup>15</sup> the increased proliferation in cultures containing CD4<sup>+</sup>CD25<sup>+</sup> from our IPEX patients suggests lack of anergy. Therefore, the CD4<sup>+</sup>CD25<sup>+</sup> cells from our patients have a functional impairment, being both unable to suppress and to remain anergic. Taken together, our findings suggest that, even if not affecting the forkhead domain, these novel FOXP3 mutations are relevant clinically and functionally and imply that other domains, besides the forkhead domain, play a role in the FOXP3 function.

In summary, we have reported 2 patients with IPEX presenting with early onset enteropathy without T1D or other endocrinopathies and a mild clinical phenotype. These patients carry novel mutations of the FOXP3 gene that do not abrogate the function of the forkhead domain and have defective Treg cells.

## References

1. 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.
2. Powell BR, Buist NR, Stenzel P. An X-linked syndrome of diarrhea, polyendocrinopathy and fatal infection in infancy. *J Pediatr* 1982;100:731–737.
3. Mirakian R, Richardson A, Milla PJ, et al. Protracted diarrhoea of infancy: evidence in support of an autoimmune variant. *BMJ* 1986;293:1132–1136.
4. Gambineri E, Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. *Curr Opin Rheumatol* 2003;15:430–435.
5. Chatila TA, Blaeser F, Ho N, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J Clin Invest* 2000;106:R75–R81.
6. Schubert LA, Jeffery E, Zhang Y, et al. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J Biol Chem* 2001;276:37672–37679.
7. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurphy mouse. *Nat Genet* 2001;27:68–73.
8. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 2005;6:331–337.
9. Sakaguchi S. Naturally arising Foxp3-expressing CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;6:345–352.
10. Lamioni A, Parisi F, Isacchi G, et al. The immunological effects of extracorporeal photopheresis unraveled: induction of tolerogenic dendritic cells and regulatory T cells in vivo. *Transplantation* 2005;79:846–850.
11. Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999;11:1185–1194.
12. Guimaraes CP, Lemos M, Menezes I, et al. Characterization of two mutations in the ABCD1 gene leading to low levels of normal APDP. *Hum Genet* 2001;109:616–622.
13. Bennet CL, Brunkow ME, Ramsdell F. A rare polyadenylation signal mutation of the FOXP3 gene (AAUAAA→AAUGAA) leads to the IPEX syndrome. *Immunogenetics* 2001;53:435–439.
14. Bakke AC, Purtzer MZ, Wildin RS. Prospective immunological profiling in a case of immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). *Clin Exp Immunol* 2004;137:373–378.
15. von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 2005;6:338–344.

---

Address requests for reprints to: Dr. Fabrizio De Benedetti, IRCCS Ospedale Pediatrico Bambino Gesù, Direzione Scientifica, Piazza S. Onofrio 4, Roma, Italy 00165. e-mail: debenedetti@opbg.net; fax: (39) 06-68592013.

Supported by grants from the Italian Ministry of Health.