Journal of Autoimmunity 38 (2012) 49–58

Contents lists available at SciVerse ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Demethylation analysis of the *FOXP3* locus shows quantitative defects of regulatory T cells in IPEX-like syndrome

F. Barzaghi [a,b,1], L. Passerini [a,1], E. Gambineri [c], S. Ciullini Mannurita [c], T. Cornu [d], E.S. Kang [e], Y.H. Choe [f], C. Cancrini [g], S. Corrente [g], R. Ciccocioppo [h], M. Cecconi [i], G. Zuin [j], V. Discepolo [k], C. Sartirana [a], J. Schmidtko [l], A. Ikinciogullari [m], A. Ambrosi [b], M.G. Roncarolo [a,b], S. Olek [d], R. Bacchetta [a,*]

[a] *San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, 20131 Milan, Italy*
[b] *Vita Salute San Raffaele University, Via Olgettina 60, 20131 Milan, Italy*
[c] *Department of Sciences for Woman and Child's Health, Anna Meyer Children's Hospital, University of Florence, Viale Pieraccini 24, 50139 Florence, Italy*
[d] *Epiontis GmbH, Rudower Chaussee 29, 12489 Berlin, Germany*
[e] *Department of Laboratory Medicine and Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, 135-710 Seoul, Republic of Korea*
[f] *Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, 135-710 Seoul, Republic of Korea*
[g] *Department of Pediatrics, Children's Hospital Bambino Gesù and University of Rome Tor Vergata School of Medicine, P.zza S. Onofrio 4, 00165 Rome, Italy*
[h] *Clinica Medica I, Fondazione IRCSS Policlinico San Matteo, University of Pavia, Via Golgi 19, 27100 Pavia, Italy*
[i] *Human Genetics Laboratories, Ospedale Galliera, Mura delle Cappuccine 14, 16128 Genoa, Italy*
[j] *Department of Pediatrics, Vittore Buzzi Children's Hospital, Via Castelvetro 32, 20154 Milan, Italy*
[k] *European Laboratory for the Investigation of Food Induced Diseases (ELFID), Department of Pediatrics, University Federico II, Via Pansini 5, 80131 Naples, Italy*
[l] *Service of Nephrology and Hypertension and Transplantation Center, Centre Hospitalier Universitaire Vaudois (CHUV), 1011 Lausanne, Switzerland*
[m] *Department of Pediatric Immunology and Allergy, Ankara University Medical School, 6100 Ankara, Turkey*

ARTICLE INFO

Article history:
Received 9 September 2011
Received in revised form
15 December 2011
Accepted 19 December 2011

Keywords:
IPEX syndrome
IPEX-like syndrome
Regulatory T (Treg) cells
Forkhead box p3 (*FOXP3*)
Treg cell-specific-demethylated-region (TSDR)
Autoimmune enteropathy
Primary immunodeficiency (PID)

ABSTRACT

Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked (IPEX) syndrome is a unique example of primary immunodeficiency characterized by autoimmune manifestations due to defective regulatory T (Treg) cells, in the presence of *FOXP3* mutations. However, autoimmune symptoms phenotypically resembling IPEX often occur in the absence of detectable *FOXP3* mutations. The cause of this "IPEX-like" syndrome presently remains unclear.

To investigate whether a defect in Treg cells sustains the immunological dysregulation in IPEX-like patients, we measured the amount of peripheral Treg cells within the $CD3^+$ T cells by analysing demethylation of the Treg cell-Specific-Demethylated-Region (TSDR) in the *FOXP3* locus and demethylation of the T cell-Specific-Demethylated-Region (TLSDR) in the *CD3* locus, highly specific markers for stable Treg cells and overall T cells, respectively.

TSDR demethylation analysis, alone or normalized for the total T cells, showed that the amount of peripheral Treg cells in a cohort of IPEX-like patients was significantly reduced, as compared to both healthy subjects and unrelated disease controls. This reduction could not be displayed by flow cytometric analysis, showing highly variable percentages of $FOXP3^+$ and $CD25^+FOXP3^+$ T cells. These data provide evidence that a quantitative defect of Treg cells could be considered a common biological hallmark of IPEX-like syndrome. Since Treg cell suppressive function was not impaired, we propose that this reduction *per se* could sustain autoimmunity.

1. Introduction

Primary immunodeficiencies are usually due to effector T (Teff) cell impairment and manifest with defective immune responses to pathogens. However, autoimmune manifestations are often present or can even prevail over recurrent infections when the genetic defect affects regulatory T (Treg) cells, which are the major players

* Corresponding author. Tel.: +39 2 26434669; fax: +39 2 26434668.
E-mail address: rosa.bacchetta@hsr.it (R. Bacchetta).
[1] These authors equally contributed to the manuscript.

0896-8411
doi:10.1016/j.jaut.2011.12.009

in maintaining peripheral tolerance [1]. This cell subset is impaired in Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked (IPEX) syndrome, a disease caused by mutations in forkhead box p3 (*FOXP3*) gene, the master switch for the function of Treg cells [2]. Affected males show life threatening autoimmune manifestations, including severe neonatal enteropathy and eczematous dermatitis, frequently associated with type 1 diabetes mellitus (T1DM) and elevated IgE serum levels [3–5].

Notably, in about one third of patients, with clinical symptoms closely resembling IPEX syndrome, *FOXP3* is not mutated. These patients are here referred to as IPEX-like [6–10]. Most frequently, the underlying genetic defect leading to IPEX-like syndrome, if present, remains unknown, thus limiting both diagnosis and therapeutic strategies. Phenotypic manifestations similar to those reported for scurfy mice, the natural *foxp3* mutants, have been found also in mice with genetic defects affecting different molecules involved in Treg cells signalling and function, such as IL-2Rα, IL-2, CTLA-4 and STAT5 [11–14]. In humans, a small number of patients with causative mutations in *CD25* [7,15] or *STAT5b* [16–19] have been reported and most likely these genetic defects cannot account for the majority of IPEX-like cases.

While in IPEX patients Treg cells, although present, are functionally impaired [4,8,20,21], the contribution of an altered Treg cell compartment in the pathogenesis of IPEX-like syndromes remains elusive. Reduced proportion of peripheral *FOXP3* expressing T cells, as determined by flow cytometric analysis, and their reduced inhibitory activity have been recently reported in two IPEX-like patients under immune suppressive treatment [8], whereas, in another work describing a single IPEX-like patient, a low FOXP3$^+$ cell amount at flow cytometric analysis but preserved suppressive function was shown [9]. Indeed, peripheral Treg cell detection and quantification in humans has been so far limited by the fact that the main markers for their identification, CD25 and FOXP3, are also expressed by activated Teff cells [2,22,23], which can be increased in inflammatory conditions [22,24], typically occurring in autoimmune diseases. Also, the use of CD127 as additional marker to discriminate Treg cells from activated Teff cells does not allow precise definition of the Treg cell subset by phenotypic analysis, since Teff cells down-regulate CD127 upon activation [24]. Recently, a highly conserved CpG enriched element, located in the 5′ untranslated region (5′UTR) of *FOXP3* has been identified as the Treg cell-specific-demethylated-region (TSDR), since it is constantly demethylated exclusively in Treg cells [25]. Demethylation of the TSDR is associated with high and stable expression of *FOXP3* [26–28] and quantification of the cells carrying demethylated TSDR allows to measure the peripheral proportion of *bona fide* Treg cells vs Teff cells, that maintain a fully methylated TSDR [25,29]. The reliability of this method for the detection of Treg cells in peripheral blood and tissues for clinical purposes has already been demonstrated in cancer patients [29,30].

In this study we quantified Treg cells in the peripheral blood of IPEX-like patients by analysing the demethylation status of the TSDR. In parallel, we evaluated the amount of CD3$^+$ T cells by an approach analogous to the TSDR analysis using a specifically demethylated area in the intergenic regions of the *CD3*δ and *CD3*γ genes, a region we here refer to as "T-lymphocyte-specific-demethylated-region" (TLSDR) [30]. Using these methods, we were able to evaluate the relative proportion of peripheral Treg within the total T cells in these patients. Our findings show a quantitative defect in the Treg cell compartment, suggestive of decreased peripheral tolerance in IPEX-like disease. The TSDR demethylation assay showed good specificity and sensitivity, thus candidating this method as a potential tool for quantification of Treg cells in the peripheral blood of patients with immune dysregulation, alternative or complementary to the flow cytometric analysis.

2. Material and methods

2.1. Patients

Twenty-eight IPEX-like patients (23 males and 5 females) with clinical manifestations resembling IPEX in absence of *FOXP3* mutations, were enrolled for this study (samples collected from 2006 to 2010). Clinical and laboratory parameters allowed exclusion of other monogenic diseases, such as Wiskott Aldrich syndrome, Omenn's syndrome, Hyper IgE syndrome and autoimmune lymphoproliferative syndrome, which should be always considered in the differential diagnosis. Patients presented at least one of the main clinical features of IPEX syndrome, autoimmune enteropathy and/or type 1 diabetes mellitus, associated with one or more of the following: dermatitis, autoimmune thyroiditis, autoimmune haemolytic anemia, autoimmune thrombocytopenia, nephropathy, autoimmune hepatitis, alopecia, hyper IgE (with or without eosinophilia). The age of the patients ranged from 2 months to 38 years (median 5 years). All patients were investigated for the presence of mutations in *FOXP3* and found to be negative. In two patients, a homozygous point mutation of *CD25* was found. The clinical details of 23 patients were available and are summarized in Table 1. Molecular and clinical details of subjects with IPEX syndrome, included as a control group in Fig. 3C, are described elsewhere [4] (Pt 3, 9, 11, 12, 14). Twenty-seven patients (non-lymphopenic, both male and female) with well defined autoimmune and autoinflammatory diseases, different from IPEX-like syndrome, have been included as pathologic controls (type 1 diabetes mellitus, $n = 10$; celiac disease, $n = 9$; Crohn's disease, $n = 8$). Forty healthy subjects (HS), aged between 7 months and 32 years (median 9 years), were analysed in parallel. The group of HS was equally distributed among males ($n = 21$) and females ($n = 19$); since no difference was detected in Treg cell percentage, even when normalized to CD3 (not shown), males and females were pooled in a single control group. Peripheral blood was obtained from patients and HS upon informed consent in accordance with the ethical committee of San Raffaele Scientific Institute approval (protocol TIGET02 and TIGET PeriBlood).

2.2. TSDR and TLSDR demethylation analysis

Genomic DNA for TSDR analysis was isolated using the QIAamp DNA micro kit (Qiagen) from peripheral blood. Bisulfite treatment of genomic DNA, subsequent TSDR- and TLSDR-specific RT-PCR and quantitative evaluation of methylated and unmethylated *FOXP3* and *CD3* DNA were performed as previously described by Wieczorek G. et al. and by Sehouli J. et al., respectively [29,30]. Bisulfite conversion was performed using Qiagen Epitect, following the manufacturer's instructions and the efficiency of conversion was always over 99%, tested according to Lewin et al. [31]. TSDR demethylation analysis evaluates the methylation status of nucleated cells in the peripheral blood, discriminating Treg (TSDR-demethylated) from all non-Treg (TSDR-methylated) cells. Hence, Treg cell amount is expressed as percentage of total nucleated cells of the peripheral blood. Based on TLSDR analysis a patient is considered lymphopenic if CD3-expressing cell percentage on the total nucleated peripheral blood cells is below 14.9% (i.e. the minimum value detected in the cohort of HS included in the present study).

2.3. Flow cytometric analysis

Anti-CD4 and -CD25 mAbs (BD Pharmingen) were used for surface staining. Intracellular staining for FOXP3 (clone 259D,

Table 1
Clinical findings in the cohort study of IPEX-like patients.

#	Age	Onset	GI symptoms	Endocrinopathy	Skin	Infections	Other	Therapy	L
1[a]	5y	1m	Enteropathy, VA	Thyroiditis	Psoriasiform dermatitis, Alopecia universalis	Chronic CMV infection	No	FK506	↓
2	3y 7m	2y	Enteropathy	No	No	No	Nephropathy, Thrombosis	PD, AZA	↓
3	2y	5m	Enteropathy, VA, Vomiting	No	No	Sepsis	Hypothyroidism (uncertain origin)	PN	N
4	10y	na	Enteropathy	No	Erythrodermia	No	Nephrotic syndrome	AZA	N
5	2m	2m	Enteropathy, Vomiting	No	No	No	Eosinophilia	TPN	N
6	18y	2y	Enteropathy, VA	T1DM	Esfoliative dermatitis, Alopecia, fragile nails	Sepsis	Glomerulonephritis, Thrombocytopenia, AHA, A. hepatitis, Vasculitis	Steroids, FK506	↓
7	4y	6m	Enteropathy	T1DM	Eczema	No	No	No	↓
8	4m	2m	Enteropathy	No	No	No	No	IS	↓
9	3m	2m	Enteropathy	Thyroiditis	Eczema	Sepsis	Abnormal CNS seizures, Abnormal EEG findings	Ig IV	↓
10	3m	2m	Enteropathy, VA	No	No	No	No	MPD, CS	N
11	16y	7y	Intermittent enteropathy	No	Nodular eritema	RRI	Chronic nephritis	Steroids, AZA	↓
12	7y	4m	Enteropathy	No	Bullous pemphigoid	No	AHA, A. hepatitis, arthritis	Steroids, Rituximab, MMF, Cx	↓
13	5y	5m	Enteropathy, Vomiting	T1DM	Vitiligo	No	No	Past steroids, TPN	N
14	20y	Neonatal	Enteropathy	No	Mild eczema	RRI	A. hepatitis, Failure to thrive	FK506	N
15	1y 9m	9m	Enteropathy	Thyroiditis	Atopic dermatitis	CMV colitis, Otitis, pneumonia	No	PD, AZA	N
16	2m	Neonatal	Enteropathy	No	No	No	No	MPD	↓
17	10y	6y	Ulcerative colitis	T1DM	Urticaria-like	Recurrent otitis, Tonsillitis	Hypogammaglobulinemia, Partial IgAD	5-ASA	N
18	2y 5m	1y	No	T1DM	Eczema, Alopecia	No	Growth retardation, Hyper IgE	No	N
19	38y	19y	Enteropathy	T1DM, thyroiditis	Psoriasis	Recurrent foruncolosis	IgAD, AAG, AT	PD	N
20	34y	10y	Enteropathy	No	Vitiligo, Alopecia	Recurrent infections (upper airways, sinusitis, pneumonias, meningitis)	Hypogammaglobulinemia, AAG	Ig IV	N
21	33y	17y	Ulcerative colitis	No	Parapsoriasis	No	Ankylosing spondylitis, A. colangytis, interstitial pneumonia, hyper IgE	PD	N
22[a]	4m	20d	Enteropathy	T1DM, Thyroiditis	Mild eczema	CMV infection, Pneumonia	AHA, lymphadenopathy, CNS bleeding, Axial hypotonia, Failure to thrive, Hyper IgE, Hyper IgG, IgA	Ig IV	N
23	1y 5m	6m	Enteropathy, VA, Gastric and colonic ulcers	No	No	No	Hyper IgE, Eosinophilia, Food allergy	MPD	N

GI: gastrointestinal; VA: villous atrophy; T1DM: Type 1 diabetes mellitus; CMV: cytomegalovirus; RRI: recurrent respiratory infections; A: autoimmune; CNS: central nervous system; AHA: autoimmune haemolytic anemia; IgAD: IgA deficiency; AAG: autoimmune atrophic gastritis; AT: autoimmune thrombocytopenia; FK506: tacrolimus; PD: prednisone; AZA: azathioprine; PN: parenteral nutrition; TPN: total parenteral nutrition; IS: immunosuppressive drugs; IV: intravenous; MPD: methylprednisone; CS: corticosteroid; MMF: mycophenolate mophetyl; Cx: cyclophosphamide; 5-ASA: mesalazine; L: lymphocytes count; na: not available; N: normal.

[a] Patient with *CD25* mutation.

BioLegend) was performed following the manufacturer's instructions. Samples were acquired on BD FACSCanto and analysed with FCS Express Pro Software Version 3 (De Novo Software).

2.4. T cell purification and suppression assays

PBMC were purified over Ficoll-hypaque gradients. Treg cells were isolated either by FACS-sorting of $CD4^+CD25^{bright}$ T cells or magnetic bead sorting by negative selection of $CD4^+$ T cells followed by positive selection of $CD25^+$ T cells and were over 90% pure (Miltenyi Biotec). To evaluate Treg cell suppressive activity, PBMC, used as responders, were stained with CFSE (Molecular Probes), following the manufacturer's instructions, and activated in the presence of Treg Inspector coated beads (Miltenyi Biotec). Suppressor cells were added at a variable ratio and the percentage of divided responders after six days of co-culture was calculated by gating on $CD4^+$ and/or $CD8^+$ cells, as elsewhere described [32]. The suppressive function of Patient #15 Treg cells was evaluated as follows: allogeneic $CD4^+CD25^-$ T cells selected by magnetic bead sorting were used as responders, activated in the presence of

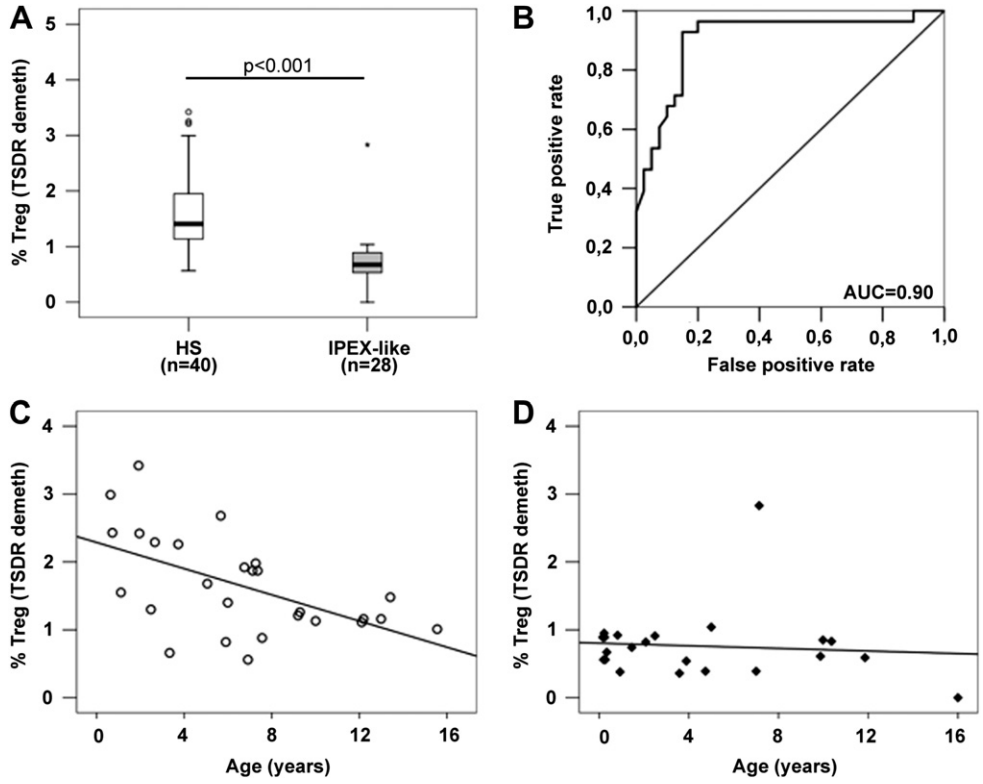

Fig. 1. TSDR demethylation analysis in the peripheral blood of IPEX-like patients. (A) Comparison between median Treg percentages (measured by TSDR demethylation assay), in the peripheral blood of HS (white bar, $n = 40$) and IPEX-like patients (grey bar, $n = 28$). Middle line indicates the median. The box represents 50% of all events and the whiskers extend to 95%. Statistical analysis was performed with Mann–Whitney *U*-test. (B) ROC curve describing the performance of TSDR assay in discriminating patients and HS. AUC value reflecting the discrimination between groups is indicated. (C, D) Scatter plot and linear regression between the percentage of Treg cells and age in healthy paediatric controls ($n = 27$), and IPEX-like paediatric patients ($n = 22$), all under 16 years.

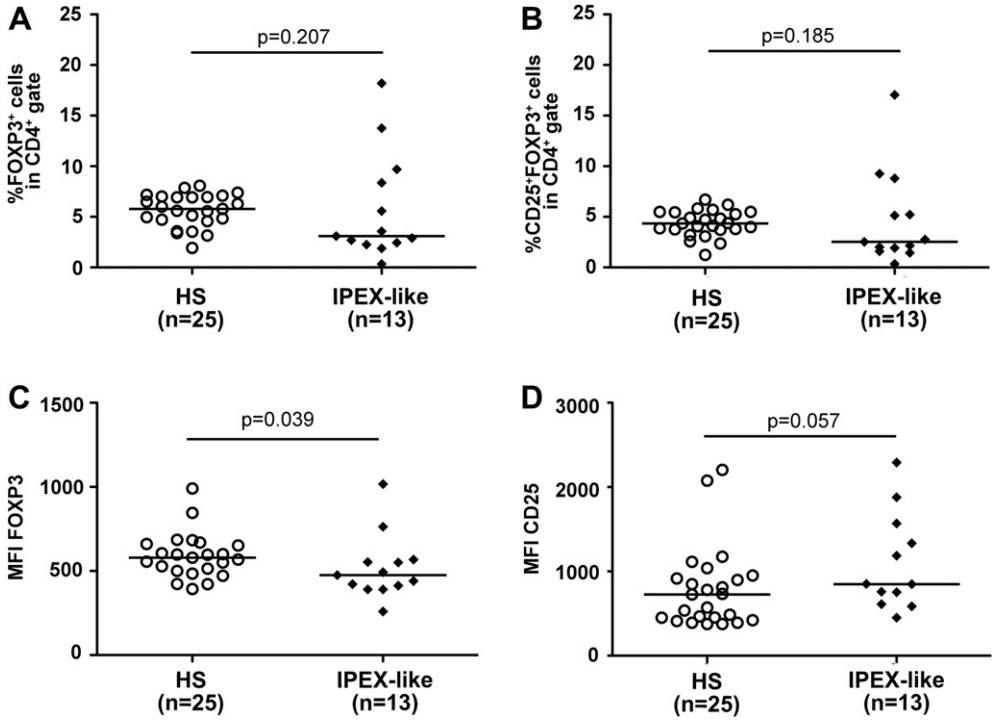

Fig. 2. Flow cytometric analysis of Treg cells in the peripheral blood of IPEX-like patients. *FOXP3*$^+$ cells were detected by flow cytometry in the peripheral blood of IPEX-like patients ($n = 13$) and HS ($n = 25$). Percentage of FOXP3$^+$ T cells in the (A) CD4$^+$ and (B) CD4$^+$CD25$^+$ T cell gates are plotted in the graphs. Mean fluorescence intensity of marker expression in (C) CD4$^+$FOXP3$^+$ and (D) CD4$^+$CD25$^+$ T cells is also shown. Differences between groups were verified by means of Mann–Whitney *U*-test.

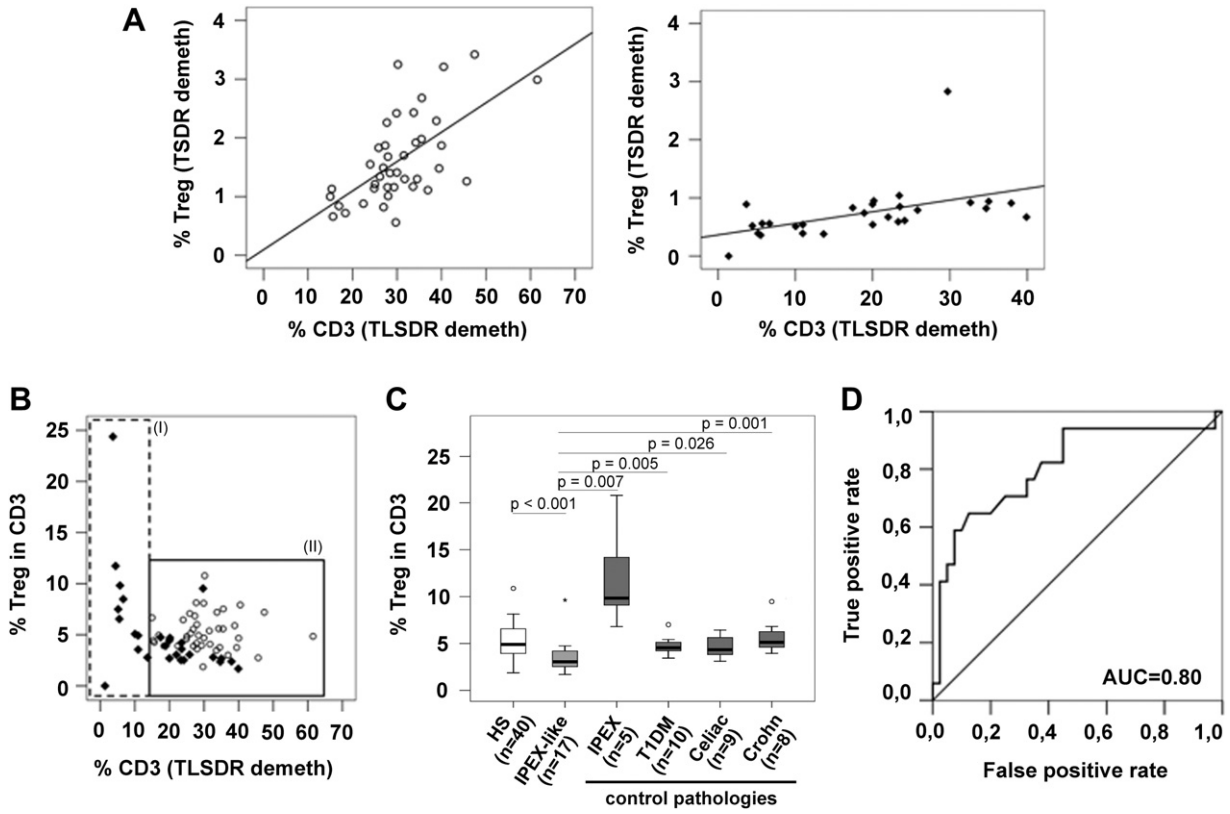

Fig. 3. Treg cell normalization to CD3$^+$ T cell counts. (A) Robust regression plots of the % of Treg cells vs the % of CD3-expressing cells (as measured by TSDR and TLSDR assays) in the peripheral blood of HS (left panel) and IPEX-like patients (right panel) are shown. Pearson correlation values were 0.617 ($p < 0.001$) for HS and 0.476 ($p = 0.010$) for patients. (B) The percentage of Treg cells normalized to TLSDR was plotted versus the CD3$^+$ cell counts, as measured by TLSDR assay. Region (I) comprises lymphopenic patients, in whom the % of CD3-expressing cells in whole blood was below 14.9%; region (II) identifies patients with lymphocyte cell counts within normal ranges. (C) The box-plots show the % of Treg normalized to CD3$^+$ T cell amount (as measured by TSDR and TLSDR combined analysis) in HS (white bar) and non-lymphopenic IPEX-like (light grey) and control patients (dark grey). Control pathologies include: IPEX syndrome ($n = 5$), T1DM ($n = 10$), Celiac ($n = 9$) and Crohn ($n = 8$) diseases. Middle lines represent the median. Statistical analysis was performed with Mann–Whitney *U*-test, $p < 0.05$ was considered significant. (D) The ROC curve describes the performance of the combined TSDR/TLSDR assays in discriminating HS and IPEX-like patients. AUC value reflecting the discrimination between groups is indicated.

allogeneic antigen presenting cells (APC), irradiated at a dose of 30 Gy and anti-CD3/CD28 mAb, and proliferation was evaluated by ^{3}[H]-thymidine incorporation after 5 days of co-culture with Treg cells, as elsewhere described [20].

2.5. Statistical analysis

Results are presented as median and range. Differences between groups were analysed with the non-parametric Mann–Whitney *U*-test and the relationship between numeric variables by means of Pearson correlation index, as indicated in the text and figure legends. To take into account possible outliers, we estimated a robust regression linear model by means of high breakdown point MM-type estimators.

For the quantitative evaluation of the *FOXP3* methylation-based discrimination of IPEX-like patients vs HS and patients with other disease controls, the area under the "receiver operating characteristics" (ROC) curve (AUC) value and its associated *p*-value were used. This is equivalent to the probability that a randomly selected IPEX-like patient shows a lower percentage of *bona fide* Treg (TSDR-demethylated) cells in peripheral blood than a randomly selected normal control or pathologic control. No discriminative ability corresponds to an AUC of 0.5. A perfect discrimination has an AUC of 1.0. All the analyses were performed with SPSS and R environment. *p*-values lower than 0.05 were considered significant.

3. Results

3.1. Clinical manifestations in IPEX-like patients

As described in Table 1, the onset of the disease ranged from 1 month (patient #1) to 19 years (patient #19), with only 4/23 patients showing a neonatal disease onset (patients #1, #14, #16 and #22). The most frequent clinical feature in the present cohort of IPEX-like patients was enteropathy, which occurred at variable age. In 20/23 patients the enteropathy involved the small bowel with histological findings including variable degree of villous atrophy and/or a polymorphic inflammatory infiltrate, and usually presenting as watery diarrhoea and weight loss, unresponsive to any dietary restriction, including gluten free diet, elemental enteral diet or total parenteral nutrition. An infectious origin of the enteropathy was excluded in all but three patients (#1, #15 and #22). In two of them early onset CMV infection was associated with CD25 deficiency, which did not allow persistent clearance of the virus. Moreover, in three patients (#17, #21 and #23), there was evidence of colonic inflammation, while in other two cases (#19 and #20) an autoimmune atrophic gastritis was found. Remarkably, serological screening for celiac disease and autoimmune enteropathy with detection of anti-transglutaminase and anti-enterocyte auto-antibodies, respectively, resulted negative.

Skin involvement was the second most common sign (16 patients out of 23), although with a wide spectrum of characteristics. In most patients the eczematous lesions were similar to atopic or

severe psoriasiform dermatitis and erythrodermia, vitiligo, nodular eritema, bullous pemphigoid and urticaria-like lesions could be also present. Notably, alopecia affected 4 patients (#1, #6, #18, #20).

T1DM was diagnosed in 7 out of 23 patients and in 6 of them it was associated with enteropathy.

Additional disease features included: i) other autoimmune disorders (in 7/23) such as thyroiditis, cytopenias, hepatitis, cholangitis, ankylosing spondylitis; ii) immunological disorders, such as IgA deficiency (2/23), IgG deficiency (2/23), and slightly to markedly increased levels of IgE (3/23) and eosinophils (1/23); iii) recurrent and severe infections (10/23).

According to the severity of enteropathy and of autoimmune manifestations, immunosuppressive therapy (IS) was administered in 15/23 patients achieving partial control of the clinical picture. Eight patients did not require any systemic IS, they received at least hormone replacement, immunoglobulins, antibiotics and supportive therapy.

Taken together, these findings indicate that patients affected with IPEX-like syndrome can be identified on the basis of the same clinical features used for the diagnosis of IPEX syndrome, including enteropathy, T1DM and skin disease, although IPEX-like may include female patients. The main difference lies in the greater variability of these symptoms in IPEX-like patients, in terms of age at onset and severity. Indeed, the onset can occur in infants or in young adults, intestinal manifestations can involve also the stomach and the colon, skin lesions occur not only as eczema but also with a wide range of clinical and histological findings. In addition, multiple autoimmune disorders appear especially with increasing age. Finally, unlike IPEX syndrome, the IPEX-like disease has a slower progression and it is better controlled by IS therapy. Patients with CD25 deficiency represent an exception to this latter observation, since their clinical manifestations may be as severe and acute as in IPEX syndrome.

3.2. A quantitative defect of Treg cells is identified by TSDR demethylation analysis in IPEX-like patients

The amount of Treg cells in the peripheral blood was determined by TSDR demethylation analysis and it is expressed as percentage of nucleated cells. Results showed a median Treg cell amount of 0.67% in IPEX-like patients ($n = 28$; range from 0 to 2.83%) and of 1.40% in HS ($n = 40$; range from 0.56 to 3.42%) (Fig. 1A). These data indicate that, taken as a whole, IPEX-like patients have a significantly reduced percentage of peripheral Treg cells, as compared to HS ($p < 0.001$). Importantly, analysis of the ROC curve showed an AUC of 0.90 ($p < 0.001$). Considering a cut-off value of 1.07% Treg cells (the value minimizing the distance between the curve and the upper left corner of the graph), the test has a sensitivity of 96% and a specificity of 80%, thus supporting the observation that TSDR analysis discriminates IPEX-like patients from HS (Fig. 1B).

Since the absolute number of lymphocytes physiologically decreases with age [33], we further analysed the amount of Treg cells across the different ages. Indeed, in pediatric HS ($n = 27$; ≤16 years of age; Treg range 0.56%–3.42%; median 1.48%) a significant age-dependent decrease of Treg cells was observed (Pearson correlation $= -0.556$, $p = 0.003$) (Fig. 1C). On the contrary, in pediatric IPEX-like patients, we did not observe any decrease in Treg cell amount in relation to age ($n = 22$; ≤16 years of age; Treg range 0%–2.83%; median 0.70%) (Fig. 1D). In adult IPEX-like patients ($n = 6$; >16 years of age) and HS, Treg cell amount did not correlate with age (not shown), but the Treg percentage was confirmed to be consistently lower in patients (from 0.51% to 0.94%; median 0.60%) than in HS (from 0.72% to 3.25%; median 1.34%). Thus, in paediatric and adult IPEX-like patients, Treg cells are constantly reduced as compared to HS regardless of age.

3.3. Flow cytometric analysis may not detect a peripheral Treg cell defect

In 13/28 IPEX-like patients TSDR demethylation could be evaluated in parallel to the flow cytometric analysis for Treg cell quantification. Results showed that the percentages of $FOXP3^+$ and $CD25^+FOXP3^+$ T cells, in gated $CD4^+$ T cells, ranged from 0.3 to 18% (median = 3%) and from 0.3 to 17% (median = 2.5%), respectively (Fig. 2A, B; Table 2). In HS ($n = 25$), $FOXP3^+$ T cells ranged from 2 to 8% (median = 5.7%) and $CD25^+FOXP3^+$ T cells from 1.2 to 6.6% (median = 4.3%) (Fig. 2A, B). Thus, according to flow cytometric analysis, median values obtained in IPEX-like patients were not significantly lower than those detected in HS ($p = 0.207$ for $FOXP3^+$ T cells and $p = 0.185$ for $CD25^+FOXP3^+$ T cells). However, the former group had a highly variable amount of *FOXP3* expressing T cells, whereas HS displayed a more narrow distribution of $FOXP3^+$ T cells (Fig. 2A, B). Addition of the negative Treg cell marker CD127 to the analysis did not reveal any significant difference in the size of the $FOXP3^+$ T cell population of IPEX-like patients as compared to HS (data not shown). These results indicate that, despite the reduced Treg cell amount detected by TSDR analysis, the frequency of FOXP3 expressing cells measured by FACS analysis is not decreased in the peripheral blood of IPEX-like patients as compared to healthy donors. Analysis on a per-cell base (mean fluorescence intensity, MFI) revealed a weaker FOXP3 expression (in the $CD4^+FOXP3^+$ T cell gate) in patients as compared to HS ($p = 0.039$), suggestive of the presence of $FOXP3^+$ activated Teff cells, rather than Treg cells, in which FOXP3 expression is stably high [24]. CD25 MFI was not significantly different in patients and HS ($p = 0.057$) (Fig. 2C, D). Overall, these data highlight a discrepancy between results obtained by the TSDR demethylation analysis and the flow cytometric data. This discrepancy is mainly evident in patients with high frequency of $FOXP3^+$ and $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell gate (Table 2). Thus, TSDR demethylation assay is more accurate than the flow cytometric analysis in the detection of peripheral Treg cells, especially when there is an overall increase of activated T cells.

3.4. Treg cell quantitative defect is confirmed by analysis of Treg cells within the $CD3^+$ T cell compartment, as determined by TLSDR analysis

In order to clarify whether the Treg cell number measured by TSDR analysis could be influenced by the amount of CD3-

Table 2
Parallel evaluation of Treg cell counts by TSDR demethylation analysis, $FOXP3^+$ and $FOXP3^+CD25^+$ T cells percentage by flow cytometric analysis and MFI in IPEX-like patients.

#	Treg (%)	$FOXP3^+$ cells (% in $CD4^+$ gate)	FOXP3 MFI	$CD25^+FOXP3^+$ cells (% in $CD4^+$ gate)	CD25 MFI
5	0.89	8.36	568	5.21	na
9	0.89	18.19	413	17.05	1569
10	0.95	5.58	391	5.14	754
11	0	3.58	259	2.75	850
13	1.04	13.75	553	8.81	451
16	0.56	1.88	422	1.59	1334
17	0.83	2.70	440	1.92	2292
18	0.91	3.09	763	2.53	587
19	0.54	2.90	493	2.15	1879
20	0.67	2.26	390	2.01	848
21	0.94	9.70	1017	9.25	1185
22	0.67	2.45	550	1.45	759
23	0.74	0.35	476	0.35	610

na: not available.

expressing T cells, the Treg cell count was normalized to that of $CD3^+$ cells, as determined by TLSDR demethylation assay. As shown by Pearson correlation analysis, both HS and IPEX-like patients showed a direct correlation between the total T cell and Treg cell percentage (Pearson correlation was 0.476 in patients, $p = 0.010$, and 0.617 in HS, $p < 0.001$). However, Treg cells in IPEX-like patients did not maintain the same ratio to CD3-expressing T cells, as in HS (Fig. 3A). Robust regression analysis revealed two different patterns with slope 0.03 and 0.50 for patients and HS, respectively; p-value for the difference of the slopes was <0.001 (1.27e-05).

In HS, $CD3^+$ T cell distribution by TLSDR analysis ranged from 14.9% to 61.5% (median = 29.5%), whereas two groups of IPEX-like patients could be identified: one comprising lymphopenic patients ($n = 11$) with TLSDR < 14.9% (i.e. the minimum value detected in HS), and a second group with TLSDR comparable to that of HS. The percentage of Treg cells within the $CD3^+$ T cell population resulted highly variable in the group of lymphopenic patients (region (I), Fig. 3B), whereas it was more homogeneous in non-lymphopenic patients ($n = 17$, region (II), Fig. 3B). Overall, in the latter group a significant reduction of Treg cells could be detected (Fig. 3C), as compared to HS (Treg % in $CD3^+$ T cells median and range were: 3.05 and 1.68–9.53 in IPEX-like; 4.89 and 1.87–10.78 in HS; $p < 0.001$). We could not detect different distribution of patients with or without IS therapy (not shown). The same analysis performed in IPEX patients ($n = 5$), who are not lymphopenic, showed that Treg cells, although *FOXP3*-mutated, were present in higher amount than those detected in IPEX-like patients (Treg % in $CD3^+$ T cells median and range: 9.84 and 6.79–20.79; $p = 0.007$) (Fig. 3C). As additional pathological controls we measured the percentage of Treg cells in $CD3^+$ T cells in patients affected with diseases whose clinical features are also present in IPEX-like syndrome, but with clear different diagnosis, i.e. patients with T1DM (median and range: 4.53% and 3.43–6.90%, respectively), celiac disease (median and range: 4.32% and 3.12–6.44%, respectively) and Crohn's disease (median and range: 5.13% and 3.96–9.38%, respectively). In all three groups the frequency of Treg cells was not different from that detected in HS (T1DM: $p = 0.522$; celiac disease: $p = 0.269$; Crohn's disease: $p = 0.492$) and it was significantly higher as compared to IPEX-like patients (T1DM: $p = 0.005$;celiac disease: $p = 0.026$; Crohn's disease: $p = 0.001$).

Notably, statistical evaluation of the results obtained from HS and IPEX-like patients through the ROC curve, showed 80% probability to distinguish between IPEX-like patients and HS (AUC = 0.80, $p<0.001$) based on the percentage of Treg/CD3 determined by TSDR and TLSDR analysis. Considering a cut-off value of 3.75% Treg cells in the T cell compartment, we obtained a sensitivity of 87%, a specificity of 65% (11 out of 17 patients showed a percentage of Treg cells below the cut-off), and an accuracy of 78% (Fig. 3D). Similarly, the TSDR/TLSDR assay showed 82% probability to distinguish IPEX-like patients from patients with different pathologies with overlapping symptoms (AUC = 0.82; Supplementary Fig. 1). Thus, also the combined demethylation analysis of TSDR and TLSDR is a valid method allowing discrimination of IPEX-like patients from HS, patients with *FOXP3* mutations and patients with different autoimmune and autoinflammatory diseases.

3.5. Treg cells from IPEX-like patients display preserved suppressive function

In order to investigate whether Treg cells from IPEX-like patients retained suppressive function, $CD4^+CD25^+$ Treg cells were purified from the peripheral blood of four patients (patients #15, 16, 17 and 20) and tested *in vitro* to evaluate their capacity to inhibit the proliferation of allogeneic and/or autologous responder cells (Fig. 4). Treg cells from all patients tested displayed preserved suppressive activity. With the exception of patient #16, suppressive activity was comparable to that of control Treg cells tested in parallel versus both $CD4^+$ and $CD8^+$ T cells from HS, used as responder cells ($n = 3$) ($p > 0.05$, Wilcoxon signed rank test). Inhibitory capacity was also maintained when patients' PBMC were used as responder cells ($n = 3$, for patients' $CD4^+$ responder cells and $n = 2$ for patients $CD8^+$ responder cells) (Fig. 4A and B). Further functional characterization of patients' Treg cells revealed that, similar to those of HS, $CD4^+CD25^+$ T cells from patients were hypoproliferative (not shown). These results indicate that Treg cell suppressive function in patients with IPEX-like syndrome is fully preserved.

4. Discussion

IPEX-like syndrome is a heterogeneous nosological entity that remains mostly of unknown origin, although specific primary immunodeficiencies can be distinguished therein, such as genetic defects in *CD25* [7,15]. In the present study, we examined a cohort of 28 patients with clinical features resembling IPEX syndrome in the absence of *FOXP3* mutations. We show that the large majority of these IPEX-like patients have low frequency of peripheral Treg cells, detected with high sensitivity and specificity by TSDR demethylation assay. Thus, we provide evidence that a quantitative defect of Treg cells can be considered a biological hallmark of IPEX-like syndrome.

For the first time, we describe a large cohort of patients presenting with IPEX-like syndrome, in which, similarly to IPEX, autoimmune enteropathy is the most frequent clinical feature together with skin lesions and other autoimmune manifestations, such as T1DM. However, unlike in IPEX, in IPEX-like syndrome the enteropathy has a variable age of onset and also females can be affected. Skin involvement is mostly characterized by eczematous lesions, although a wide spectrum of skin pathologies can be observed. Other autoimmune symptoms comprise thyroiditis, cytopenias, hepatitis, cholangitis, atrophic gastritis, and ankylosing spondylitis. Immunological disorders such as IgA deficiency, hypogammaglobulinemia, and increased serum IgE levels can be present, together with high eosinophil counts. Despite several clinical similarities with IPEX syndrome, which justify the general definition as IPEX-like, the course of the disease is less severe and patients show better therapy response.

In the attempt to investigate the pathogenesis of the disease, we and others have questioned whether the Treg compartment is affected as in IPEX syndrome. Previous studies, using flow cytometric analysis led to insufficient results to support the hypothesis of a Treg cell reduction [8,9]. These data include an evaluation of Treg cells, previously performed in five IPEX-like patients (all under IS therapy), among whom, three were diagnosed with low $CD25^+CD127^{dim}FOXP3^+$ T cell counts, and two others had a normal proportion of these cells [8, 9].

In the present cohort of IPEX-like patients, using the highly Treg-specific TSDR demethylation assay [34], we were able to identify a consistent quantitative deficiency of Treg cells. The defect could not be detected by flow cytometry that showed, on the contrary, variable results. The discrepancy between demethylation and flow cytometric analysis was particularly evident in patients with the highest number of $CD4^+FOXP3^+$ T cells (patient #9 and #21, who had a low amount of TSDR-demethylated cells). These data suggest that, in the presence of activated Teff cells, transiently expressing CD25 and FOXP3 [24,35], flow cytometric analysis loses specificity to quantify peripheral *bona fide* Treg cells. This limitation is more likely to occur in patients with ongoing autoimmunity and, therefore, high amount of circulating activated T cells. Thus,

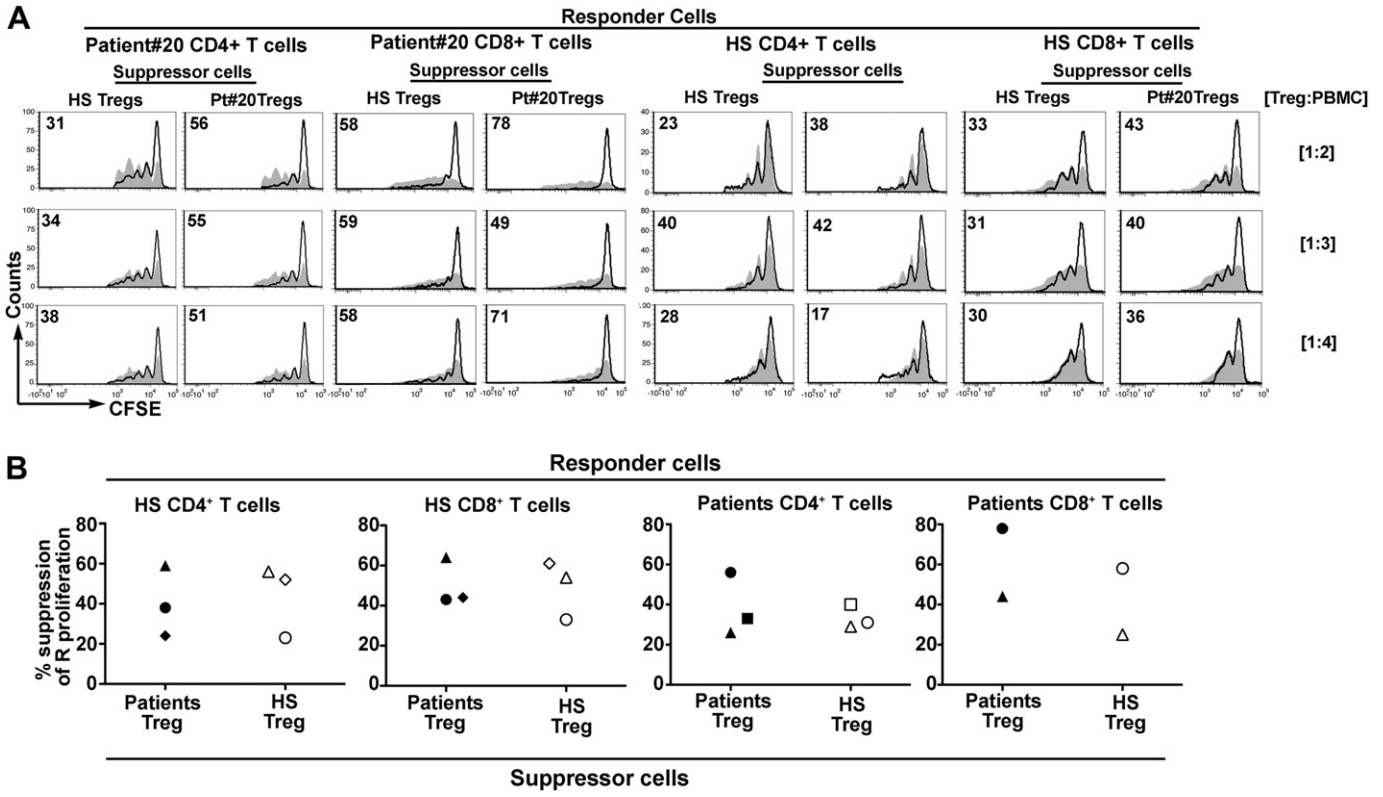

Fig. 4. Suppressive function of Treg cells isolated from the peripheral blood of IPEX-like patients. $CD4^+CD25^+$ T cells isolated from the peripheral blood of IPEX-like patients were used as suppressor cells in a suppression assay, using allogeneic and/or autologous PBMC as responders. $CD4^+CD25^+$ T cells isolated from the peripheral blood of one HS were tested in parallel and used as control. (A) Histograms of CFSE dilution of one representative patient (#20) are shown. Analysis was performed by gating either on $CD4^+$ or on $CD8^+$ responder T cells. Numbers in the plots indicate percent inhibition. The relative [suppressor: PBMC responder] cell ratio is also indicated. (B) Average %inhibition of proliferation versus the indicated responder cells is plotted in the graphs. The highest [suppressor: responder] cell ratio tested is plotted for each patient. Black symbols refer to patients, white symbols to HS tested in parallel. ●: Patient #20; ▲: Patient #17; ■: Patient #15; ◆: Patient #16.

especially in these conditions, TSDR demethylation analysis emerges as a key complementary method to evaluate *bona fide* Treg cell amount. Moreover, the combined analysis of TSDR and TLSDR demethylation allows quantification of Treg cells within the total amount of T cells, so that Treg cells can be studied in lymphopenic and non-lymphopenic patients. Indeed, in non-lymphopenic IPEX-like patients, the low number of Treg cells was confirmed, suggesting that autoimmunity could be sustained by an unbalanced Treg/T cell ratio, with consequent insufficient amount of Treg cells to control pathogenic Teff cells. Furthermore, our results demonstrate that the Treg cell reduction is a feature specific for patients with IPEX-like syndrome, since it is not detected in patients with other autoimmune pathologies of different origin. On the contrary, a different aetiology of the autoimmunity can be hypothesized in lymphopenic patients, in whom the reduction of Treg cells is associated with an overall decreased amount of $CD3^+$ T cells.

Statistical evaluation of results through the ROC curve showed that both methods have high probability to discriminate between IPEX-like patients and HS: 90% (AUC = 0.90) considering the TSDR demethylation analysis alone, and 80% (AUC = 0.80) using the combined TSDR/TLSDR demethylation analysis. The latter assays also allow the distinction between IPEX-like patients and other pathologic controls (AUC = 0.82). Hence, parallel demethylation analysis of TSDR and TLSDR identifies a biological parameter, alternative or complementary to flow cytometry, which permits precise Treg cell quantification and exclusion of concomitant lymphopenia, often characterizing primary immunodeficiencies. In addition, demethylation analysis can be performed with low amount of biological material (i.e. less than 200 μL of peripheral blood), allowing highly reproducible quantification of Treg cells even in infants, in whom blood sampling is limited.

Despite reduced in number, Treg cells of IPEX-like patients displayed preserved suppressive function in all patients tested. Previous studies reported that Treg cell in vitro function in patients with autoimmune enteropathy, without *FOXP3* mutations could vary from normal to reduced in relationship with the level of FOXP3 expression [8,9]. Interestingly, all the patients that we tested had low frequency of Treg cells as measured by TSDR assay. When the percentage of Treg cells was normalized by TLSDR, only in two out of four patients (patients #15 and 20), both with fully preserved *in vitro* suppressive activity, the Treg cell frequency was below the cut-off value, thus suggesting that in these patients autoimmunity may indeed be due to reduced Treg cell frequency. In one patient (patient #16) the overall low Treg cell number was due to presence of lymphopenia, as demonstrated by TLSDR assay. In this case the quantitative assay is not informative, since Treg cells were not specifically undersized with regard to the Teff cell compartment. In addition, their function resulted partially defective, thus leaving the possibility that in this patient the pathology could be due to a complex defect including the functional impairment of Treg cells alone or rather of multiple T cell subsets. In the last patient tested (patient #17) both Treg cell number and function were comparable to healthy subjects, thus indicating that the defect underlying the disease is likely to affect the effector rather than the regulatory T cell compartment, as also indicated by the partial resistance to suppression of patient's responder cells. Overall, our functional

results still confirm a certain heterogeneity within IPEX-like patients, although clearly identifies a cohort in which the quantitative Treg defect is present. Our approach allows the rapid and precise identification of this subgroup of patients, who would potentially benefit from innovative therapies based on the adoptive transfer of *ex-vivo* expanded autologous Treg cells.

The reduction of Treg cells has been observed in other autoimmune diseases [36,37], but the causes of this reduction still need to be clarified. The unbalance between the Treg/Teff compartments may be due either to defective homeostatic proliferation of the peripheral Treg cell pool as compared to the conventional T cell pool, or to impairment of peripheral Treg cell survival. Alternatively, Treg cells may be reduced due to impaired thymic development and/or migration to periphery. Further studies on patients affected with IPEX-like syndrome are required to identify the biological/molecular cause of the Treg cell decrease.

Overall, we propose TSDR and TLSDR demethylation analysis as reliable tools for the quantitative evaluation of Treg cells in patients with immune dysregulation, allowing the identification of a defect otherwise not detectable in IPEX-like patients, integrating flow cytometric evaluation, especially in the presence of inflammation, and standardizing the quantification of Treg cells. Our analysis provides new insights into the pathogenesis of at least a subset of IPEX-like patients, in whom autoimmunity can be ascribed to the unbalance between the Treg/Teff compartments, thus paving the way for the development of targeted therapies.

Disclosure statement

S.O. has declared a financial interest in a Company whose potential product was used in the present work. All other authors have no conflicting financial interests.

Acknowledgements

This work was supported by the Italian Telethon foundation (Grant Tele10A4 and GGP07241 to R.B.), by the Seventh framework programme (FP7) of the European Community for research, technological development and demonstration activities (Cell-PID to R.B.), and by the Italian Ministry of Health (Grant RF-2009-1485896 to R.B.). The Authors are grateful to Prof. G.R. Corazza, Clinica Medica I, Fondazione IRCCS Policlinico San Matteo, Università degli Studi di Pavia, Italy, for his clinical expertise. The Authors also thank M. Battaglia, G. Giamporcaro and A. Valle from the Diabetes Research Institute, San Raffaele Scientific Institute, Milan, Italy for help in collecting samples from diabetic patients.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jaut.2011.12.009.

References

[1] Westerberg LS, Klein C, Snapper SB. Breakdown of T cell tolerance and autoimmunity in primary immunodeficiency–lessons learned from monogenic disorders in mice and men. Curr Opin Immunol 2008;20:646–54.
[2] Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity 2005;22:329–41.
[3] Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 2002;39:537–45.
[4] Gambineri E, Perroni L, Passerini L, Bianchi L, Doglioni C, Meschi F, et al. Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: inconsistent correlation between forkhead box protein 3 expression and disease severity. J Allergy Clin Immunol 2008;122:1105–12. e1.
[5] Ochs HD, Gambineri E, Torgerson TR. IPEX, FOXP3 and regulatory T-cells: a model for autoimmunity. Immunol Res 2007;38:112–21.
[6] Yong PL, Russo P, Sullivan KE. Use of sirolimus in IPEX and IPEX-like children. J Clin Immunol 2008;28:581–7.
[7] Caudy AA, Reddy ST, Chatila T, Atkinson JP, Verbsky JW. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. J Allergy Clin Immunol 2007;119:482–7.
[8] Moes N, Rieux-Laucat F, Begue B, Verdier J, Neven B, Patey N, et al. Reduced expression of FOXP3 and regulatory T-cell function in severe forms of early-onset autoimmune enteropathy. Gastroenterology 2010;139:770–8.
[9] Zuber J, Viguier M, Lemaitre F, Senee V, Patey N, Elain G, et al. Severe FOXP3+ and naive T lymphopenia in a non-IPEX form of autoimmune enteropathy combined with an immunodeficiency. Gastroenterology 2007;132:1694–704.
[10] Ruemmele FM, Moes N, de Serre NP, Rieux-Laucat F, Goulet O. Clinical and molecular aspects of autoimmune enteropathy and immune dysregulation, polyendocrinopathy autoimmune enteropathy X-linked syndrome. Curr Opin Gastroenterol 2008;24:742–8.
[11] Sharma R, Zheng L, Deshmukh US, Jarjour WN, Sung SS, Fu SM, et al. A regulatory T cell-dependent novel function of CD25 (IL-2Ralpha) controlling memory CD8(+) T cell homeostasis. J Immunol 2007;178:1251–5.
[12] Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 1993;75:253–61.
[13] Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008;322:271–5.
[14] Snow JW, Abraham N, Ma MC, Herndier BG, Pastuszak AW, Goldsmith MA. Loss of tolerance and autoimmunity affecting multiple organs in STAT5A/5B-deficient mice. J Immunol 2003;171:5042–50.
[15] Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. Proc Natl Acad Sci U S A 1997;94:3168–71.
[16] Kofoed EM, Hwa V, Little B, Woods KA, Buckway CK, Tsubaki J, et al. Growth hormone insensitivity associated with a STAT5b mutation. N Engl J Med 2003;349:1139–47.
[17] Bernasconi A, Marino R, Ribas A, Rossi J, Ciaccio M, Oleastro M, et al. Characterization of immunodeficiency in a patient with growth hormone insensitivity secondary to a novel STAT5b gene mutation. Pediatrics 2006;118:e1584–92.
[18] Hwa V, Little B, Adiyaman P, Kofoed EM, Pratt KL, Ocal G, et al. Severe growth hormone insensitivity resulting from total absence of signal transducer and activator of transcription 5b. J Clin Endocrinol Metab 2005;90:4260–6.
[19] Cohen AC, Nadeau KC, Tu W, Hwa V, Dionis K, Bezrodnik L, et al. Cutting edge: decreased accumulation and regulatory function of CD4+ CD25(high) T cells in human STAT5b deficiency. J Immunol 2006;177:2770–4.
[20] Bacchetta R, Passerini L, Gambineri E, Dai M, Allan SE, Perroni L, et al. Defective regulatory and effector T cell functions in patients with FOXP3 mutations. J Clin Invest 2006;116:1713–22.
[21] d'Hennezel E, Ben-Shoshan M, Ochs HD, Torgerson TR, Russell LJ, Lejtenyi C, et al. FOXP3 forkhead domain mutation and regulatory T cells in the IPEX syndrome. N Engl J Med 2009;361:1710–3.
[22] Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006;24:209–26.
[23] Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 2007;37:129–38.
[24] Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol 2007;19:345–54.
[25] Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. Eur J Immunol 2007;37:2378–89.
[26] Polansky JK, Schreiber L, Thelemann C, Ludwig L, Kruger M, Baumgrass R, et al. Methylation matters: binding of Ets-1 to the demethylated Foxp3 gene contributes to the stabilization of Foxp3 expression in regulatory T cells. J Mol Med 2010;88:1029–40.
[27] Nagar M, Vernitsky H, Cohen Y, Dominissini D, Berkun Y, Rechavi G, et al. Epigenetic inheritance of DNA methylation limits activation-induced expression of FOXP3 in conventional human CD25-CD4+ T cells. Int Immunol 2008;20:1041–55.
[28] Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. Blood 2009;114:3727–35.
[29] Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. Cancer Res 2009;69:599–608.
[30] Sehouli J, Loddenkemper C, Cornu T, Schwachula T, Hoffmuller U, Grutzkau A, et al. Epigenetic quantification of tumor-infiltrating T-lymphocytes. Epigenetics 2010;6:236–46.
[31] Lewin J, Schmitt AO, Adorjan P, Hildmann T, Piepenbrock C. Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates. Bioinformatics 2004;20:3005–12.
[32] Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods 1994;171:131–7.

[33] van Gent R, van Tilburg CM, Nibbelke EE, Otto SA, Gaiser JF, Janssens-Korpela PL, et al. Refined characterization and reference values of the pediatric T- and B-cell compartments. Clin Immunol 2009;133:95–107.
[34] Janson PC, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. FOXP3 promoter demethylation reveals the committed Treg population in humans. PLoS One 2008;3:e1612.
[35] Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983–90.
[36] Barreto M, Ferreira RC, Lourenco L, Moraes-Fontes MF, Santos E, Alves M, et al. Low frequency of CD4+CD25+ Treg in SLE patients: a heritable trait associated with CTLA4 and TGFbeta gene variants. BMC Immunol 2009;10:5.
[37] Badami E, Sorini C, Coccia M, Usuelli V, Molteni L, Bolla AM, et al. Defective differentiation of regulatory FoxP3+ T Cells by small-intestinal dendritic cells in patients with type 1 diabetes. Diabetes 2011;60:2120–4.