

Letter to the Editor

Forkhead box protein 3 (FOXP3) mutations lead to increased T_H17 cell numbers and regulatory T-cell instability

To the Editor:

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a primary immunodeficiency with severe autoimmunity caused by mutations in the forkhead box protein 3 gene (*FOXP3*). *FOXP3* encodes a transcription factor critical for the function of naturally occurring regulatory T (Treg) cells, which are devoted to tolerance maintenance. Impairment of Treg cells is the main pathogenic mechanism underlying IPEX syndrome.¹ However, increasing evidence suggests that *FOXP3* might also impinge effector T (Teff) cells.² Indeed, *FOXP3* inhibits T_H17 cell differentiation and IL-17 production,³ and we reasoned that in patients with IPEX syndrome, in addition to loss of suppressive function, augmented T_H17 responses might be responsible for autoimmunity.

We therefore investigated the presence of IL-17-producing T cells both in patients with new-onset IPEX (age <2 years; within 9 months from onset; n = 6), and in long-term patients (age >12 years; n = 4), who were undergoing immunosuppression. The present cohort was also heterogeneous in terms of site of the mutation (see Table E1 in this article's Online Repository at www.jacionline.org). After *in vitro* short-term expansion (12–20 days) in the presence of immobilized anti-CD3 (10 µg/mL) and soluble anti-CD28 (1 µg/mL) mAbs and recombinant human IL-2 (100 U/ml), patients' CD4⁺ T cells displayed significantly increased IL-17A and decreased IFN-γ production compared with that seen in cells of healthy donors (HDs; Fig 1, A and B), whereas IL-10 production was comparable with that seen in control T-cell lines (data not shown). In addition, activated PBMCs from patients with new-onset disease (n = 3) showed high IL-17A production (range in patients, 57–1493 pg/mL; range in HDs, 0–80 pg/mL; results were normalized to 10⁶ cells/mL), whereas the production of T_H1 (IFN-γ) and T_H2 (IL-5) cytokines was in the same range as that seen in HDs (not shown). In long-term patients overall cytokine production by PBMCs was low, but production of IL-17A was augmented in the presence of recombinant human IL-2 (fold increase ranging from 1.4 to 37, data not shown). Treatment with multidrug immunosuppression is likely to alter the T-cell capacity to produce cytokines, including IL-17, but prompt restoration in the presence of exogenous IL-2 suggests that T_H17 cells are present also in long-term patients, although they are hyporesponsive because of immunosuppression.

Analogously, we found increased expression of the T_H17 cell-specific transcription factor retinoic acid-related orphan receptor variant 2 (*RORC2*) in *ex vivo* CD4⁺ T cells of patients with new-onset disease compared with that seen in age-matched HDs (Fig 1, C). This increase was specific because it was not paralleled by high expression of the T_H1 cell transcription factor T-bet and was not due to a higher frequency of memory/effector CD45RO⁺ T cells in patients (not shown). In long-term patients *RORC2* expression was not significantly augmented compared with that seen in HDs (data not shown). Overall, these findings indicate that T_H17 cells are present in the peripheral blood of patients with IPEX syndrome, regardless of the type or site of mutation, especially at early onset, whereas T_H1 and T_H2 cytokine levels

are comparable with those seen in HDs. This sheds light on previous controversial data on IL-17 production in patients with IPEX syndrome, which either describe high IL-17 production at onset in 1 patient together with increased T_H1 and T_H2 cytokine levels⁴ or report IL-17 production within normal ranges in patients undergoing immunosuppression.⁵

Although Treg cells were initially thought to bear a stable phenotype, recent advances in the field revealed new features for this lineage. The possibility that specific subsets of Treg cells can diverge from a regulatory fate and acquire proinflammatory properties, including the capacity to produce T_H17 cytokines, has been proposed as a physiologic mechanism to allow efficient response to pathogens or to restrain detrimental immune regulation.⁶ On the other hand, it has been suggested that in murine models Treg cells are capable of acquiring Teff cell functions and might become autoreactive, although the latter phenomenon is still controversial.⁶ In this context IPEX syndrome could serve as a sensible model to investigate Treg cell plasticity based on the hypothesis that Treg cells undergo thymic development but experience peripheral conversion from a regulatory to an effector (ie, IL-17-producing) phenotype in the presence of *FOXP3* mutations.

We found that T cells epigenetically imprinted as Treg cells, as measured by means of analysis of the Treg cell-specific demethylated region,⁷ were indeed present in peripheral blood of patients at levels comparable with those seen in HDs (Fig 1, D), but *FOXP3*-expressing T cells were randomly distributed among CCR6⁺CD161⁺ and CD161[−] subpopulations or even more enriched in the CD161⁺ subset. On the contrary, in HDs *FOXP3*⁺ Treg cells were mainly comprised in the CD4⁺CCR6⁺CD161[−] T-cell population (Fig 1, E). Therefore in patients with IPEX, CCR6⁺CD161⁺ T cells, which also include T_H17 cells, contained more *FOXP3*⁺ T cells than their counterpart from HDs (*P* = .02 vs HDs, Mann Whitney *U* test). These data demonstrate that (1) although dysfunctional, circulating putative Treg cells are present in patients with IPEX syndrome, thus indicating that wild-type *FOXP3* is not necessary for thymic differentiation of Treg cells in human subjects, and that (2) *FOXP3*-mutated Treg cells cosegregate with the CCR6⁺CD161⁺ T-cell subset, which also includes T_H17 cells, strongly suggesting their peripheral conversion from suppressor to IL-17-producing cells.

Interestingly, levels of IL-6 and IL-23, cytokines described to favor human T_H17 cell differentiation and expansion,³ were significantly increased in the sera of patients with IPEX syndrome compared with that seen in HDs (Fig 1, F), whereas levels of IL-1β were not (data not shown). In addition, in the presence of IL-1β and IL-6, T-cell clones generated from CD4⁺CD25^{bright} T cells of patients upregulated IL-17A production, whereas IL-17A production by activated HD Treg cells was low or negative and did not consistently increase in the presence of inflammatory cytokines (Fig 1, G). On the other hand, IL-17 production by HD Treg cells was specifically induced on *FOXP3* knockdown by *FOXP3*-specific small-interfering RNA (LV-siRNA-*FOXP3*)⁸ in CD4⁺CD25^{bright} Treg cell-derived lines (18.5 ± 9.4-fold increase vs LV-siRNA-control, n = 5), confirming that Treg cell stability is maintained by expression of wild-type *FOXP3* (Fig 1, H). Overall, these results indicate that *FOXP3*-mutated Treg cells are

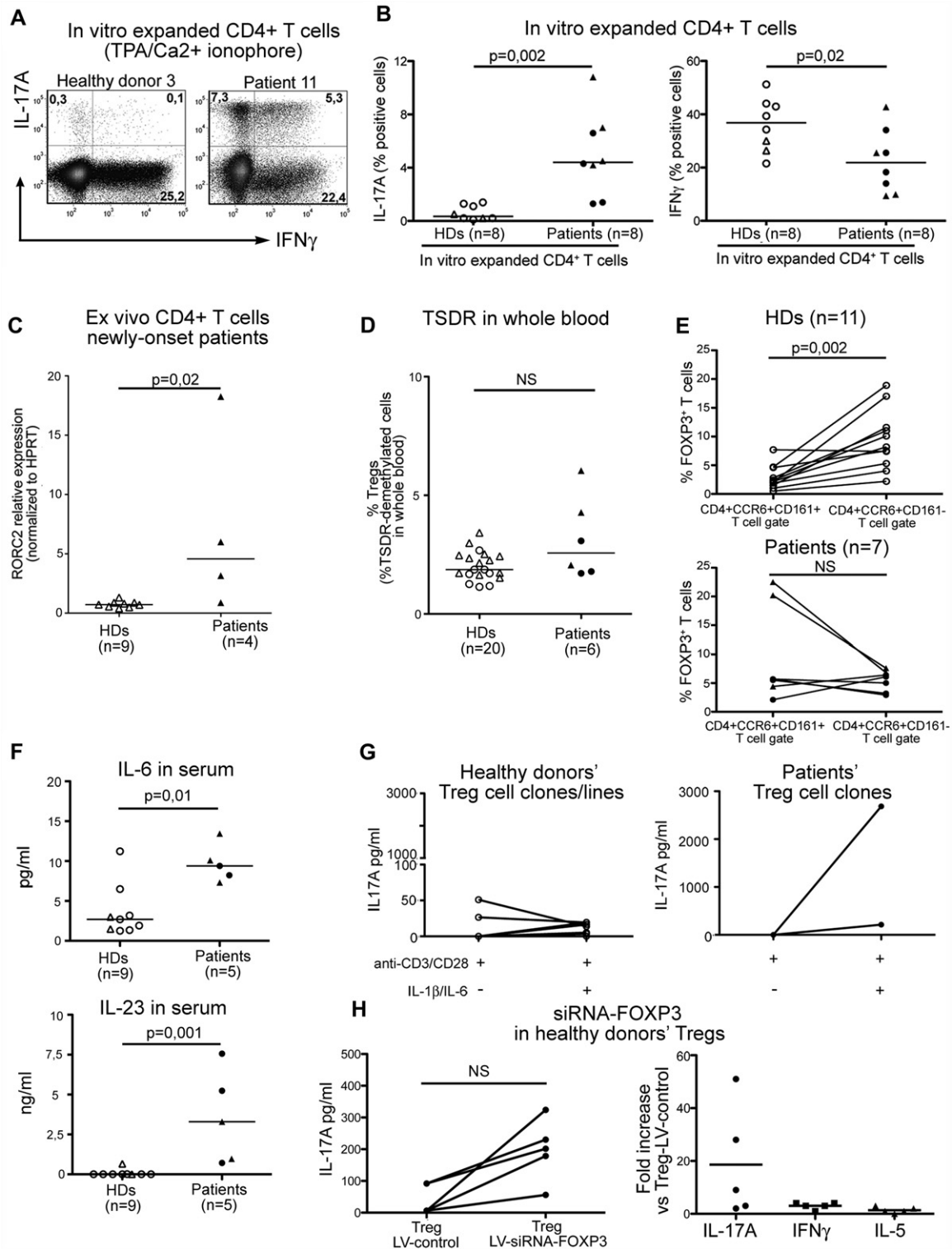


FIG 1. **A** and **B**, IL-17A/IFN- γ production by CD4⁺ T cells. **TPA**, Tetradenoyl phorbol acetate. **C**, Retinoic acid-related orphan receptor variant 2 (*RORC2*) mRNA expression. **D**, Treg cell-specific demethylated region (*TSDR*) demethylation in whole blood. **E**, FOXP3 in CD4⁺CCR6⁺CD161⁺/CD161⁻ T cells. **F**, IL-6 and IL-23 levels in sera. **G**, IL-17A production by HDs' and patients' Treg cell clones/lines. **H**, IL-17A levels on *FOXP3* knockdown. In **Fig 1**, **B-F**, circles, subjects older than 12 years; triangles, subjects younger than 2 years. The Mann-Whitney *U* test was used in all but **Fig 1**, **E/H** (Wilcoxon signed-rank test). Middle lines indicate medians. *NS*, Not significant

prone to produce IL-17 and further suggest that in the absence of functional FOXP3 and in inflammatory conditions, Treg cells in patients with IPEX syndrome can be reprogrammed to produce IL-17A. Whether the presence of *FOXP3* mutations favors *de novo* generation of IL-17-producing Teff cells remains to be clarified.

In conclusion, we provide evidence of increased frequency of IL-17-producing T cells in patients with IPEX syndrome. This phenomenon might be sustained, at least in part, by inflammation-driven conversion of dysfunctional Treg cells unable to properly regulate IL-17 production. Taking into account that a significant proportion of Treg cells are self-antigen specific,⁹ we propose that in patients with IPEX syndrome, *FOXP3*-mutated T_H17-converted Treg cells might directly contribute to the autoimmune damage seen in the target organs. Therefore studies to demonstrate the self-antigen specificity and pathogenicity of such cells should be pursued. Thus therapies for the restoration of Treg cell function might not be sufficient to control the disease unless in combination with approaches aimed at inhibiting the T_H17 shift of potentially autoreactive *FOXP3*-mutated Treg cells.

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TABLE E1. Summary of the clinical, immunologic, and mutation details of patients with IPEX

Patient no.	<i>FOXP3</i> mutation	Age (2011)	Age at onset	Enteropathy	Endocrinopathy	Skin disease	Other	Therapy
3*	IVS1+2T>G	5 y	Neonatal	Severe diarrhea with VA	IDDM	Mild eczema	Hepatitis/sepsis	HLAid BMT†
7*	IVS8+4A>G	9 y	Neonatal	Severe diarrhea with VA	IDDM	Severe eczema	Hepatitis	IS (multiple drugs)
8*	p.P339A	—	Neonatal	Severe diarrhea with VA	IDDM	—	Autoimmune hepatitis/AEA/FT hepatosplenomegaly	IS (multiple drugs)
9	p.R347H	15 y	Neonatal	Severe diarrhea with VA	IDDM	Mild eczema	Hepatitis/thrombocytopenia/Coombs negative anemia/food allergy/FT	IS (low steroids)
11	p.A384T	16 y	Neonatal	Severe diarrhea with eosinophilic infiltration without VA	IDDM thyroiditis	Severe eczema/alopecia	AEA/interstitial pneumonia/FT	IS (multiple drugs)
12*	p.F373A	9 y	Neonatal	Severe diarrhea with VA	IDDM	Eczema	—	HLAid BMT†
14	p.L242P	15 y	4 mo	Severe diarrhea with VA	—	Mild eczema	Sepsis/nephropathy	IS (multiple drugs)
17*	p.I346T	3 y	Neonatal	Severe diarrhea without VA	—	Severe seborrhoeic dermatitis	MRSA sepsis/ <i>Candida</i> species CVL infection	CB-URD HSCT†
18*	AATAAA>AA TGAA	8 y	Neonatal	Severe diarrhea with VA	—	Seborrhoeic dermatitis	Hypotonia	CB-URD HSCT†
20	IVS7+2del	28 y	5 mo	Severe diarrhea with VA	—	Eczema	Thrombocytopenia/arthritis/sepsis/bronchitis	IS (multiple drugs)

AEA, Autoimmune hemolytic anemia; BMT, bone marrow transplantation; CB-URD, cord blood-unrelated donor; CVL, central venous line; FT, failure to thrive; HLAid, HLA identical; HSCT, hematopoietic stem cell transplantation; IDDM, insulin-dependent diabetes mellitus; IS, immunosuppression; MRSA, methicillin-resistant *Staphylococcus aureus*; VA, villous atrophy.

*Patients studied before 2 years of age.

†Patients' samples used for this study were collected before transplantation.