

Point mutants of forkhead box P3 that cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked have diverse abilities to reprogram T cells into regulatory T cells

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Background: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is a primary immunodeficiency with autoimmunity caused by mutations in *forkhead box P3* (*FOXP3*), which encodes a transcription factor involved in regulatory T (Treg) cell function. The mechanistic basis for how different mutations in *FOXP3* cause distinct manifestations of IPEX remains unclear.

Objective: To determine whether 3 different point mutants of *FOXP3* that cause severe or mild IPEX differ in their ability to reprogram conventional T cells into Treg cells.

Methods: Human CD4⁺ T cells were transduced with wild-type or point mutant forms of *FOXP3*, and changes in cell surface marker expression, cytokine production, proliferation and suppressive capacity were assessed. *Ex vivo* T_H17 cells were also transduced with different forms of *FOXP3* to monitor changes in IL-17 production.

Results: The forkhead mutant F373A failed to upregulate CD25 and CCR4, did not suppress cytokine production, and induced suppressive activity less effectively than wild-type *FOXP3*. In contrast, although the forkhead mutant R347H was also defective in upregulation of CD25, it suppressed the production of cytokines, conferred suppressive capacity on CD4⁺ T cells, and suppressed IL-17 production. F324L, a mutant outside the

forkhead domain associated with mild IPEX, was equivalent to wild-type *FOXP3* in all aspects tested.

Conclusion: Mutations in *FOXP3* that cause IPEX do not uniformly abrogate the ability of *FOXP3* to regulate transcription and drive the development of Treg cells. These data support the notion that factors in addition to functional changes in Treg cells, such as alterations in conventional T cells, are involved in the pathogenesis of IPEX. (J Allergy Clin Immunol 2010;■■■■:■■■■-■■■■.)

Key words: T regulatory cells, IPEX, *FOXP3*, autoimmunity, tolerance, IL-17

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is an X-linked autoimmune disease that presents early in childhood with severe diarrhea, chronic dermatitis, hyper-IgE, and polyendocrinopathies, and is often fatal.¹⁻⁸ IPEX is caused by mutations in *forkhead box P3* (*FOXP3*), a transcription factor critical for the function of regulatory T (Treg) cells.⁹⁻¹¹ Scurfy mice, which have a null mutation in *Foxp3* and develop a lethal autoimmune disorder,^{12,13} can be cured by adoptive transfer of Treg cells,⁹ leading to the hypothesis that IPEX results from a lack of Treg cells. However, patients with IPEX generally have circulating CD4⁺*FOXP3*⁺ T cells,^{14,15} suggesting that the disease may not be exclusively attributed to a numerical deficiency of Treg cells.

FOXP3 is a forkhead family member, and in addition to its DNA-binding forkhead domain, it contains a leucine zipper that is important for homodimerization and heterodimerization, a zinc finger, and a repressor domain.¹⁶⁻²⁰ *FOXP3* acts both as a transcriptional activator and repressor—for example, at the *CD25* (*IL-2 receptor α chain*) promoter and the *IL-2* promoter, respectively.²¹⁻²³ Its effects on transcription require coordinated interactions with other proteins including nuclear factor of activated T cells^{23,24}; HIV-1 transactivator of transcription interacting protein, 60 kd²⁰; Eos²⁵; retinoic acid receptor-related orphan receptor γ (ROR- γ)^{26,27}; retinoic acid receptor-related orphan receptor α ^{27,28}; and Runt-related transcription factor 1.²⁹ Overexpression of *FOXP3* is sufficient to reprogram mouse CD4⁺ conventional T (Tconv) cells into cells that are phenotypically and functionally identical to Treg cells.^{9,11,30} Continuous and high expression of *FOXP3* can also reprogram human CD4⁺ Tconv cells into Treg cells.^{31,32} In human beings, *FOXP3* is also expressed transiently in Tconv cells on activation,³³⁻³⁷ and Tconv and Treg *FOXP3*-

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Abbreviations used

APC:	Antigen-presenting cell
ΔNGFR:	Truncated nerve growth factor receptor
FOXP3:	Forkhead box P3
[³ H]-TdR:	Tritiated thymidine
IPEX:	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
MFI:	Mean fluorescence intensity
ROR-γt:	RAR-related orphan receptor γ
Tconv:	Conventional T
Treg:	Regulatory T
TSDR:	Regulator T-specific demethylated region
WT:	Wild-type

expressing cells can be distinguished by analysis of the Treg-specific demethylated region (TSDR). In this region, CpG islands are methylated in Tconv cells and demethylated in Treg cells.³⁸⁻⁴⁴

Mutations in *FOXP3* that cause IPEX are found throughout the gene,^{4,5} but it is unclear how different mutations affect the development and function of Treg cells. To understand better the cellular and molecular basis of IPEX, we examined the impact of 3 different point mutants derived from patients with IPEX on the ability of FOXP3 to reprogram Tconv cells into Treg cells. Surprisingly, mutations in the forkhead DNA binding domain of FOXP3 that caused severe IPEX (R347H and F373A) were only partially blocked in their ability to reprogram Tconv cells into Treg cells. Moreover, a mutation in FOXP3 that caused mild disease (F324L) did not differ from wild-type (WT) FOXP3 in any aspect tested. These results challenge the notion that IPEX results solely from a defect in Treg cells.

METHODS**Patients**

Detailed clinical histories are summarized in Table I.

Construction and production of lentiviral vectors

Point mutant forms of *FOXP3* were amplified by RT-PCR from cDNA derived from peripheral blood of the patients, and a hemagglutinin tag was added at the amino terminal end. The *FOXP3b* isoform and hemagglutinin-tagged WT or point mutant forms of the full-length *FOXP3a* isoform were cloned into the bidirectional pCCL lentiviral vector.³² All vectors were confirmed by sequencing, and lentivirus was produced as described.^{32,45}

Cell purification, transduction, and culture of T-cell lines

Peripheral blood was obtained from healthy volunteers who gave written informed consent in accordance with the protocol approved by the University of British Columbia Clinical Research Ethics Board. Antigen-presenting cells (APCs), CD4⁺ T cells, CD4⁺CD25[−]CD45RO[−] (naive) T cells, and *ex vivo* Treg cells were isolated, transduced, and expanded as described.^{32,45}

Flow-cytometric analysis

Analysis of CD25, CD127, CCR4 (all BD Biosciences, Mississauga, Ontario Canada), and glucocorticoid-induced tumor necrosis factor receptor-related protein (R&D Systems, Minneapolis, Minn) was performed on resting T-cell lines. A minimum of 6, 3, 2, or 8 independent experiments was performed for CD25, CD127, GITR, or CCR4, respectively, derived from at least 2 independent donors. To stain for hemagglutinin (Roche Applied Sciences, Laval, Quebec, Canada) and FOXP3 (236/AE7; eBioscience, San Diego,

Calif), cells were fixed and permeabilized with FixPerm buffer (eBioscience) before addition of antibodies. Samples were read on a BD FACSCanto (BD Biosciences) and analyzed with FCS Express Pro Software version 3 (De Novo Software, Los Angeles, Calif).

Determination of cytokine production by transduced T cells

A total of 200,000 T cells/well were stimulated with immobilized anti-CD3 mAbs (10 μg/mL) and soluble anti-CD28 (1 μg/mL) in a final volume of 250 μL. Supernatants were collected after 20 hours (IL-2) or 48 hours (all others) and assayed using a T_H1/T_H2 cytometric bead array (BD Biosciences).

Proliferation of T cells

A total of 50,000 cells/well were plated with 50,000 irradiated (50 Gy) APCs and soluble anti-CD3 (OKT3; 0.1 or 1 μg/mL). After 72 hours, tritiated thymidine ([³H]-TdR, 1 μCi/well; GE Healthcare, Baie d'Urfe, Quebec, Canada) was added for 16 hours. Proliferation is expressed as percentage of control truncated nerve growth factor receptor (ΔNGFR) cell [³H]-TdR counts. Compiled proliferation data are the average of a minimum of 7 independent experiments derived from at least 3 donors.

Suppression of CD4⁺ or CD8⁺ T-cell proliferation and cytokine production by transduced T cells

A total of 8000 CD4⁺ T cells were activated with anti-CD3/anti-CD28 coated beads (Invitrogen, Burlington, Ontario, Canada) at a ratio of 1 bead:8 cells and cultured with increasing numbers of transduced T cells. After 6 days, wells were pulsed with [³H]-TdR for 16 hours. Suppression of IFN-γ and TNF-α production from CD4⁺ responders was measured by coculture of 50,000 CD4⁺ T cells with 50,000 irradiated (50 Gy) APCs plus soluble anti-CD3 (0.1 or 1 μg/mL) with increasing numbers of transduced T cells. Supernatants were collected after 72 hours. Data are expressed as the percent suppression of proliferation or of cytokine produced by CD4⁺ responders alone.

Compiled data are the averages of a minimum of 3 independent experiments derived from 2 donors for F324L lines or at least 3 donors for WT FOXP3, R347H, and F373A lines.

Suppression of CD8⁺ T cells was measured by labeling PBMCs with carboxyfluorescein diacetate succinimidyl ester, activating 100,000 PBMCs/well with anti-CD3/anti-CD28-coated beads (Invitrogen) at a ratio of 1 bead:32 cells, and culturing with increasing numbers of transduced T cells as indicated. After 4 days, proliferation of gated CD8⁺ T cells was determined. Data are representative of a minimum of 2 independent experiments derived from 2 donors.

Transduction, culture, and analysis of human T_H17 cells

CD4⁺CXCR3[−]CCR4⁺CCR6⁺ T cells were sorted from CD4⁺ T cells on a BD FACSAria (BD Biosciences) to >95% purity as described.⁴⁶ CD4⁺CXCR3⁺ cells were sorted as control IL-17IFN-γ⁺ T cells. Cells were activated and transduced as described and, after 12 days, stimulated with phorbol 12-myristate 13-acetate (10 ng/mL, Sigma-Aldrich, Oakville, Ontario, Canada) and ionomycin (100 ng/mL, Sigma-Aldrich) for 6 hours, with brefeldin A (10 μg/mL, Sigma-Aldrich) added for the last 4 hours. Cells were stained for ΔNGFR, fixed and permeabilized, and stained with anti-IFN-γ (BD Biosciences) and anti-IL-17A (eBioscience), and analysis was performed on gated ΔNGFR⁺ T cells.

Statistical analysis

All statistical analyses were performed with the 2-tailed Student paired *t* test, and data were transformed with log (base 10) to account for variability between the cell lines. *Error bars* represent the SEM. *Asterisks* indicate a significant difference compared with WT FOXP3. *Asterisks above a joining line* represent significant differences compared with control ΔNGFR-transduced cells (****P* < .001; ***P* = .001-.01; **P* = .01-.05).

TABLE I. Summary of the clinical manifestations, treatments, and outcomes of patients with IPEX with the FOXP3 mutations investigated

	F324L (pt 5)* ⁵	R347H (pt 9) ⁵	R347H (pt 10) ⁵	R347H (case 1) ⁷	R347H ⁸	F373A (pt 12) ⁵
Location of mutation	Outside forkhead domain	In forkhead domain				In forkhead domain
Age at onset	Neonatal	Neonatal	<1 y	<1 y	<1 y	Neonatal
Symptoms at onset	Severe diarrhea	Hyperglycemia, diarrhea	Recurrent ear infections, high IgE levels	IDDM	Gastritis	Ketoacidosis
Enteropathy	Severe diarrhea with eosinophilic infiltration without villous atrophy	Severe diarrhea with villous atrophy	Severe chronic gastritis with mucosal atrophy	Diarrhea	Severe gastritis with eosinophilic infiltration and mucosal atrophy	Severe diarrhea with villous atrophy
Endocrinopathy	—	IDDM	IDDM	IDDM	IDDM	IDDM
Skin disease	Mild eczema	Mild eczema	Mild xerosis	—	—	Eczema
Other autoimmune diseases	—	Hepatitis, thrombocytopenia	—	—	—	—
Other symptoms	Allergic asthma	Coombs-negative anemia, food allergy, failure to thrive	Pancreatic exocrine failure, failure to thrive	Failure to thrive, intractable hypertension with encephalopathy	Intestinal metaplasia, failure to thrive, steatorrhea, abnormal sweat test, pancreatic atrophy, hypo- γ -globulinemia	—
Autoantibodies	—	GAD, AIA, ICA, aETC	—	aETC	—	AIA, aETC
Treatment	None; spontaneously recovered	Immunosuppression (CsA for first 3 y, prednisone)	Immunosuppression (CsA, methyl-prednisone)	Immunosuppression (CsA, prednisone; later: tacrolimus); HLA-id BMT	Immunosuppression (prednisone, CsA)	HLA-id BMT at 9 mo
Outcome (as of July 2010 unless noted otherwise)	Alive 7 y old	Alive 14 y old	Alive 23 y old	Died at age 14 y 194 d post-BMT	Alive 22 y old as of September 2009	Alive 7.5 y old

aETC, Antienterocyte antibody; AIA, anti-insulin antibody; CsA, cyclosporin A; GAD, antiglutamate decarboxylase antibody; HLA-id BMT, HLA-identical bone marrow transplant; ICA, anti-islet cell antibody; IDDM, insulin-dependent diabetes mellitus; Pt, patient; —, unknown or not tested.

*This patient has an additional mutation/polymorphism: 543 C>T at intron 4/exon 5 boundary.

RESULTS

Effects of IPEX point mutants of FOXP3 on expression of Treg-associated cell surface proteins

To understand better whether the degree of IPEX is correlated with the relative dysfunction of FOXP3 in Treg cells, we selected 3 different point mutant forms of FOXP3 known to cause different severities of disease^{5,7,8,15} (Fig 1; Table I). To use our previously developed overexpression system whereby FOXP3 reprograms naive Tconv cells into Treg cells,^{32,45} the mutant forms of *FOXP3* were fused to a hemagglutinin tag and cloned into the pCCL lentivirus vector, which encodes Δ NGFR as a marker gene. Hemagglutinin and FOXP3 were coexpressed in transduced T cells, and the levels of expression of WT and mutant forms of FOXP3 were equal (Fig 1).

FOXP3 regulates expression of several cell-surface markers characteristic of Treg cells by acting as either a transcriptional repressor or activator.^{21–23} To determine whether IPEX point mutant forms of FOXP3 were defective in transactivation and/or repression, we analyzed the cell surface phenotype upon expression of WT or mutant FOXP3. As expected, cells transduced with WT FOXP3 upregulated CD25, GITR, and CCR4 and downregulated CD127 (Fig 2). T cells transduced with F324L did not differ significantly from those transduced with WT FOXP3 in expression of any of these proteins. In contrast, T cells transduced with F373A and R347H failed to upregulate CD25 to the same extent

as those transduced with WT FOXP3 (average fold increase in mean fluorescence intensity (MFI) compared with control was 4.95 ± 0.89 for WT FOXP3 vs 2.36 ± 0.60 for F373A, $P < .0001$, $n = 12$; and 3.17 ± 0.53 for R347H, $P = .0075$, $n = 8$). Although both F373A and R347H retained their ability to promote expression of GITR and suppress CD127 in transduced T cells, only F373A was defective at upregulating CCR4 (2.43 ± 0.33 average fold increase in MFI for WT FOXP3 compared with 1.62 ± 0.11 for F373A; $P = .0129$; $n = 10$). Although F373A and R347H did not upregulate CD25 to the same extent as WT FOXP3, they upregulated CD25 compared with control (Δ NGFR)–transduced cells ($P = .0198$, $n = 12$; and $P = .0036$, $n = 7$ for F373A and R347H, respectively). Likewise, compared with Δ NGFR-transduced cells, F373A upregulated CCR4 ($P < .0001$; $n = 10$). These data indicate that mutations in the forkhead domain of FOXP3 do not necessarily abrogate its ability to regulate transcription.

Effects of IPEX point mutants of FOXP3 on cytokine production

A hallmark of Treg cells is their inability to produce IL-2, IFN- γ or TNF- α ,^{11,47,48} so we investigated whether point mutations in FOXP3 abrogated its capacity to suppress cytokine production. As expected, WT FOXP3 transduction repressed production of

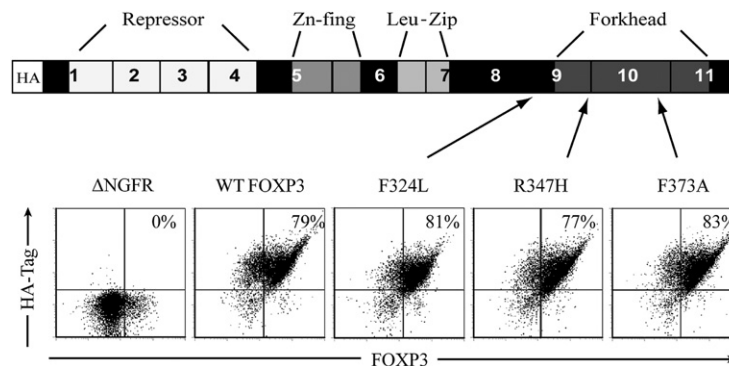


FIG 1. Expression of hemagglutinin (HA)-tagged FOXP3 IPEX mutants in Tconv cells. Expression of HA and FOXP3 after purification of transduced cells is shown for 1 representative of a minimum of 3 experiments. Locations of mutations of patients with IPEX are indicated on a schematic representation of FOXP3 that also depicts the N-terminal HA tag and exon numbers. *Zn-fing*, zinc-finger; *leu-zip*, leucine zipper.

IFN- γ , TNF- α , IL-2, IL-4, and IL-5 (Fig 3), and F324L or R347H suppressed cytokine production to the same extent. In contrast, F373A was defective at suppressing production of IFN- γ , TNF- α , IL-2, IL-4, and IL-5 ($P = .0173$, $P = .0025$, $P = .0073$, $P = .0050$, and $P = .0083$ compared with WT FOXP3, respectively; $n = 4$ -5). The repressive capacity of F373A, however, was not completely eliminated: compared with Δ NGFR-transduced cells, F373A transduction resulted in a significant decrease in the production of TNF- α and IL-4 ($P = .0287$, $n = 5$; and $P = .0200$, $n = 4$, respectively). As we previously reported, expression of WT FOXP3 did not significantly affect IL-10 production,³² and none of the point mutants differed in this respect. These data show that mutations in FOXP3 that cause IPEX do not necessarily abrogate the capacity of the protein to suppress cytokine production.

IPEX point mutant forms of FOXP3 do not uniformly lose their capacity to reprogram Tconv cells into suppressive Treg cells

We next tested whether T cells transduced with the point mutant forms of FOXP3 became hypoproliferative and acquired suppressive function. Although expression of all forms of FOXP3 resulted in decreased proliferation compared with Δ NGFR expression, the ability of the F373A and R347H mutants to induce hyporesponsiveness was reduced compared with WT FOXP3 ($P = .0019$, $n = 13$; and $P = .0034$, $n = 8$, respectively; Fig 4, A). All transduced T-cell lines proliferated in the presence of IL-2 (data not shown), demonstrating that the cells were equally viable.

As expected, T cells transduced with WT FOXP3 significantly inhibited the proliferation and cytokine production of *ex vivo* responder CD4⁺ T cells and the proliferation of *ex vivo* CD8⁺ T cells in a dose-dependent manner (Fig 4, B-D). Surprisingly, F324L and R347H mutants retained a normal capacity to confer transduced T-cell lines with the ability to suppress both proliferation and cytokine production of responders. In contrast, F373A was significantly impaired at reprogramming T cells into suppressive cells compared with WT FOXP3. T-cell lines transduced with F373A suppressed CD4⁺ T-cell proliferation by an average of $39.3\% \pm 13.5\%$ at a 1:2 ratio (transduced cells:responder cells), whereas WT FOXP3-transduced lines suppressed by an average of $82.3\% \pm 6.7\%$ at this ratio ($P = .0149$, $n = 6$; Fig 4, B). To

confirm these data further, we performed suppression assays by labeling PBMCs with carboxyfluorescein succinimidyl ester and monitoring the proliferation of CD8⁺ T cells with increasing ratios of transduced T cells (Fig 4, C). A similar trend was observed: F373A-transduced but not R347H-transduced or F324L-transduced cells had a reduced capacity to suppress the proliferation of CD8⁺ T cells compared with WT FOXP3-transduced cells.

The ability of F373A-transduced lines to suppress production of IFN- γ and TNF- α by CD4⁺ T-cell responders was also significantly diminished, while F324L and R347H-transduced cells did not differ from WT-transduced cells in this capacity (Fig 4, D). At a 1:2 ratio (transduced cells:responders), IFN- γ production by CD4⁺ responder T cells increased in the presence of F373A-transduced T cells, whereas lines transduced with WT FOXP3 suppressed IFN- γ production by CD4⁺ responders by $61.4\% \pm 8.0\%$ (F373A compared with WT FOXP3; $P = .0267$; $n = 5$). Suppression of TNF- α by F373A-transduced lines at the 1:2 ratio was $2.1\% \pm 20.5\%$ compared with $65.3\% \pm 7.1\%$ by WT FOXP3-transduced lines ($P = .0015$; $n = 3$). Hence, even though forkhead mutants F373A and R347H both cause severe IPEX, they are not equally defective at reprogramming Tconv into suppressive cells *in vitro*.

IPEX mutant forms of FOXP3 do not uniformly lose their capacity to inhibit IL-17 production from T_H17 cells

Recently, a role for FOXP3 in antagonizing the differentiation of proinflammatory T_H17 cells via protein-protein interactions with ROR- γ t and Runt-related transcription factor 1 has been recognized.^{26,27,49} Given the unexpected finding that IPEX point mutants of FOXP3 retained some or all of their ability to convert Tconv cells into Treg cells, we hypothesized that their ability to inhibit IL-17 production from T_H17 cells may be impaired. Enriched populations of *ex vivo* T_H17 cells were sorted as CD4⁺CXCR3⁺CCR4⁺CCR6⁺ T cells⁴⁶ and transduced with WT FOXP3, point mutant forms, or the alternately spliced form of FOXP3 (FOXP3b)^{26,28} that is defective at suppressing ROR- γ t-driven IL-17 production in 293T cells²⁶ because it lacks the ROR- γ t and RAR-related orphan receptor α interacting domain. Consistent with its deficiency in blocking ROR- γ t-driven IL-17,²⁶ expression of FOXP3b did not suppress IL-17 production by *ex vivo* T_H17 cells to the same extent as WT FOXP3

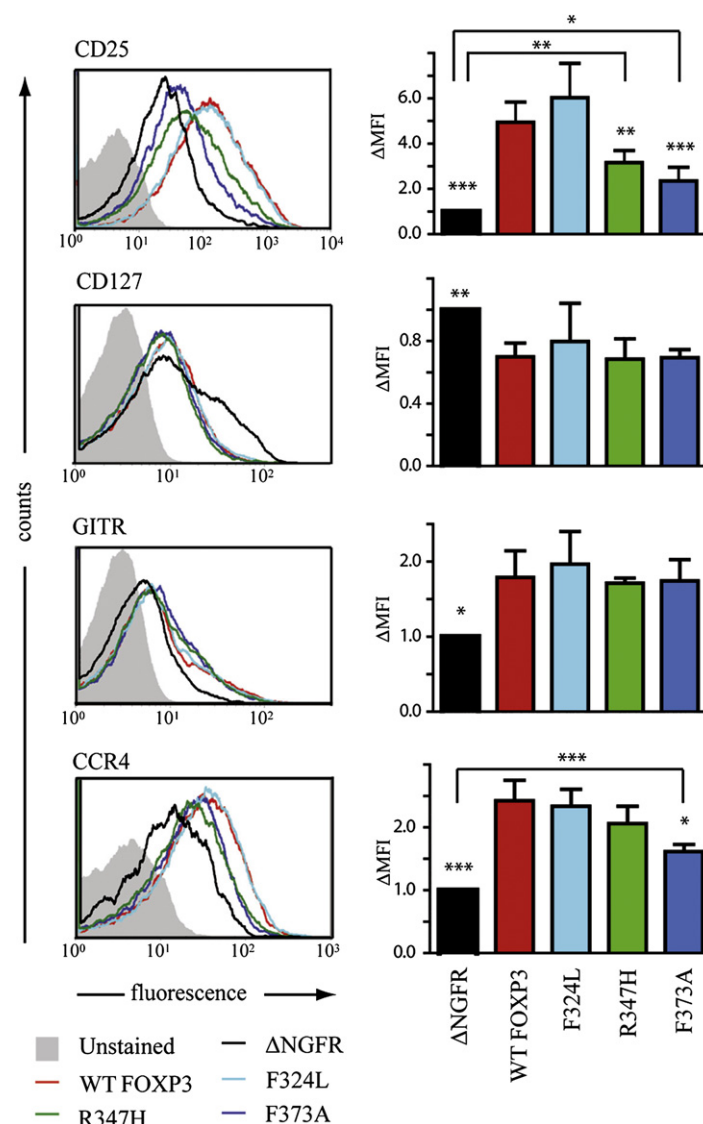


FIG 2. Cell surface marker expression on T cells transduced with WT FOXP3 or IPEX mutants. Histograms show 1 representative experiment gated on Δ NGFR⁺ cells. Bar graphs depict the average fold difference in MFI (Δ MFI) compared with the MFI of control transduced cells. Error bars represent SEM. Asterisks indicate a significant difference compared with WT FOXP3. Asterisks above a joining line represent significant differences compared with control δ NGFR-transduced cells. *** P < .001; ** P = .001-.01; * P = .01-.05.

(P = .0088; n = 3; Fig 5). Although T_H17 cells transduced with F324L or R347H were equivalent to WT FOXP3 in suppression of IL-17 production, T_H17 cells transduced with F373A had a deficiency similar to that of FOXP3b and did not suppress IL-17 production to the same extent as cells transduced with WT FOXP3. On average, F373A transduction suppressed IL-17 production by only $26.7\% \pm 19.2\%$ compared with $49.5\% \pm 12.1\%$ suppression by WT FOXP3, although this difference did not reach statistical significance (P = .1092; n = 3). These data suggest an impaired ability of F373A to regulate IL-17 expression by fully differentiated *ex vivo* T_H17 cells compared with WT FOXP3.

DISCUSSION

Evidence that even patients with IPEX with severe disease can have circulating FOXP3⁺ T cells^{14,15} suggests that the cellular

basis for the disease may be a result of a functional defect rather than a numerical loss of Treg cells. To investigate this possibility further, we examined whether 3 point mutant forms of FOXP3 were altered in their ability to reprogram Tconv cells into Treg cells. We found that the mutants were not equally impaired, and none were completely deficient, in their capacity to drive the Treg phenotype. These findings suggest some forms of IPEX may not be caused exclusively by altered Treg cell function.

FOXP3 can act as both a transcriptional activator and repressor of cell surface marker expression.²¹⁻²³ On ectopic expression of WT FOXP3, changes in gene expression characteristic of Treg cells occur, including upregulation of CD25 and CCR4 and downregulation of CD127.^{32,50} Although F373A was defective at upregulating CD25 and CCR4, it retained a normal capacity to downregulate CD127, suggesting that alternative mechanisms may regulate transcriptional repression versus activation. A critical role for the F373 residue in transactivation of genes

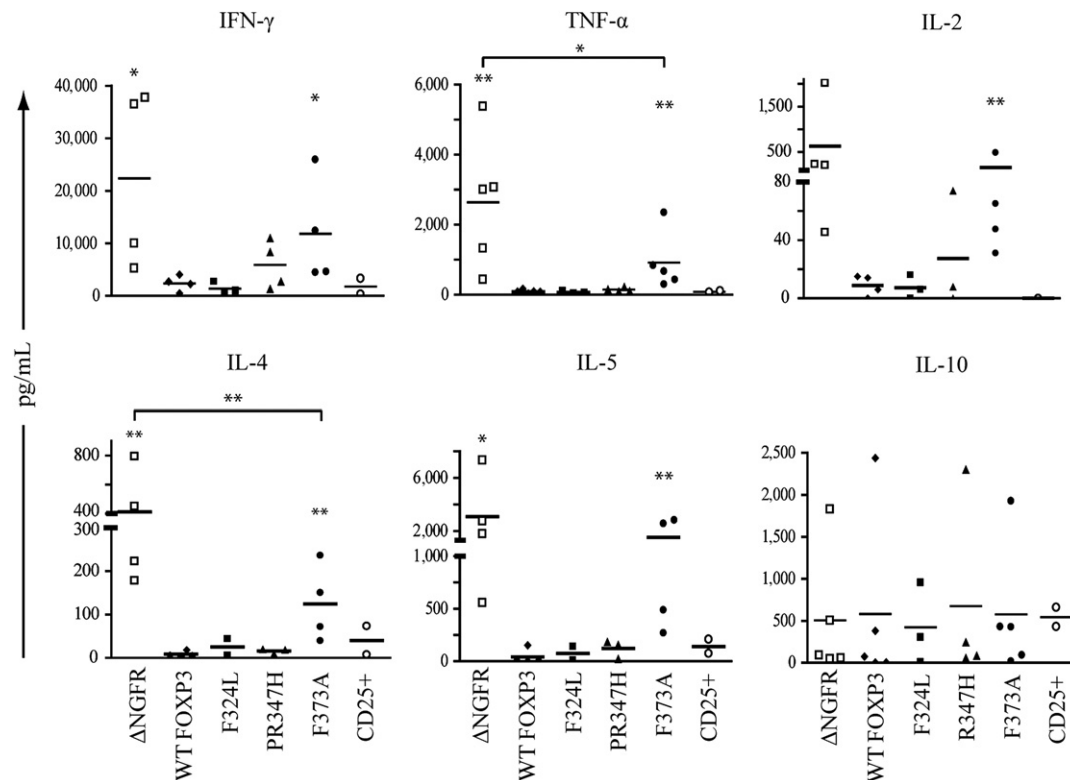


FIG 3. Cytokine production by T cells transduced with WT FOXP3 or IPEX mutants. Each point represents a single determination; horizontal bar indicates the mean. CD4⁺CD25⁺ Treg cell lines cultured in parallel to transduced cell lines are included for comparison. Asterisks indicate a significant difference compared with WT FOXP3. Asterisks above a joining line represent significant differences compared with control Δ NGFR-transduced cells. *** $P < .001$; ** $P = .001-.01$; * $P = .01-.05$.

by FOX proteins is supported by a study on mutations in forkhead box L2: a mutation in forkhead box L2 at F90, which corresponds to F373 in FOXP3, results in defective transactivation of a *forkhead box L2*-regulated promoter.⁵¹

We previously found that the expression of CD25 in *ex vivo* PBMCs from a patient with IPEX with the F373A mutation was comparable to that in PBMCs from normal donors, and CD4⁺CD25⁺ T-cell lines generated from this patient maintained high CD25 expression in the resting state.¹⁵ These data contrast with our current finding that cell lines transduced with F373A have lower expression of CD25 compared with cell lines transduced with WT FOXP3. This difference could be a result of additional Treg cell-associated transcription factors that contribute to transactivation of CD25 which are not expressed in transduced CD4⁺ T cells but are present in CD25⁺ Treg cells from patients with IPEX. This hypothesis is in line with new evidence that proteins such as the transcription factor Eos²⁵ and the surface receptor glycoprotein A repetitions predominant^{52,53} have a role in defining the Treg cell phenotype, and that cells with a Treg phenotype can develop in the absence of functional FOXP3.^{54,55}

A hallmark of Treg cells is their lack of cytokine production because of the repressive effects of FOXP3.^{11,47,48} In contrast with Scurfy mice that have elevated cytokine production,⁵⁶ PBMCs from patients with IPEX carrying either the F373A or F324L mutation have decreased cytokine production compared with normal donors.¹⁵ This difference between mice and human beings with FOXP3 mutations may exist because in human

beings, but not mice, FOXP3 is transiently expressed in activated Tconv cells.³³⁻³⁷ Although the role of FOXP3 in human Tconv cells is unknown, the surprising phenotype of decreased cytokine production in patients with IPEX suggests there could be a dominant-negative effect of mutant activation-induced FOXP3 on nuclear factor of activated T cells-regulated cytokine production in Tconv cells.

Because T_H17 cells have been implicated in many autoimmune diseases,⁵⁷ we speculated that mutant forms of FOXP3 might inadequately suppress IL-17 production. To investigate this possibility, we developed a novel assay in which sorted CXCR3⁺CCR4⁺CCR6⁺ T cells were transduced with FOXP3 and showed for the first time that WT FOXP3 can inhibit IL-17 expression in fully differentiated *ex vivo* T_H17 cells. Surprisingly, F324L and R347H were as effective as WT FOXP3 at suppressing IL-17 expression by transduced sorted T_H17 cells. By contrast, F373A was less able to suppress IL-17 production, so further investigation is warranted to determine whether deregulated IL-17 production may have contributed to disease progression in the patient carrying the F373A mutation.

FOXP3 can positively regulate its own expression,⁵⁸ raising the possibility that exogenous FOXP3 could upregulate expression of endogenous WT FOXP3, which could contribute to a Treg phenotype. Although we cannot formally exclude a possible contribution of endogenous FOXP3, analysis of the methylation state of the TSDR, the region of the FOXP3 locus that must be demethylated to allow high and stable expression of FOXP3,³⁸⁻⁴⁴ suggested this was unlikely. Tconv cells transduced with WT

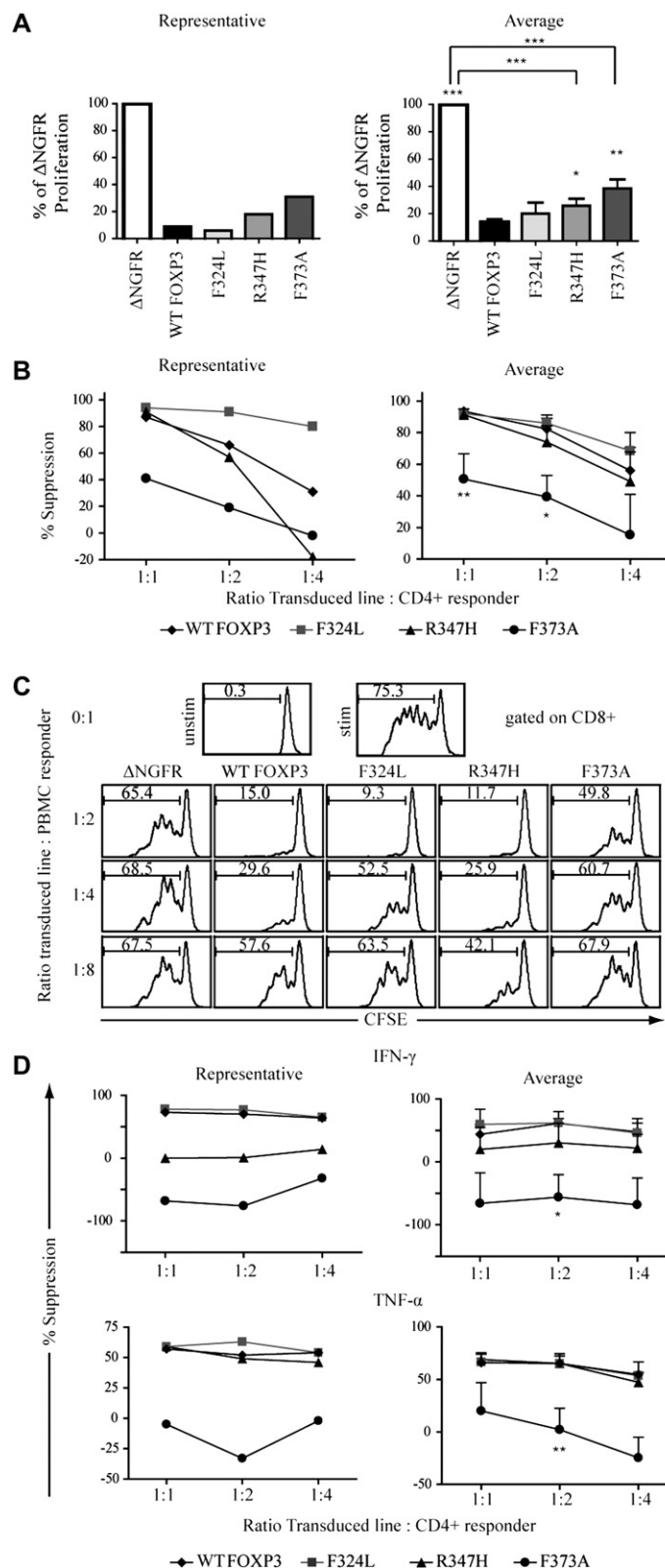


FIG 4. Proliferation and suppressive capacity of T cells transduced with WT FOXP3 or IPEX mutants. Error bars represent SEM. Asterisks indicate a significant difference compared with WT FOXP3. Asterisks above a joining line represent significant differences compared with control δ NGFR-transduced cells. *** $P < .001$; ** $P = .001-.01$; * $P = .01-.05$. **A**, Proliferation of T-cell lines. **B**, Suppression of CD4⁺ T-cell proliferation by transduced T cells determined by [³H]-TdR incorporation. **C**, Suppression of CD8⁺ T-cell proliferation by transduced T cells determined by carboxyfluorescein succinimidyl ester (CFSE) dilution. **D**, Suppression of CD4⁺ T-cell IFN- γ and TNF- α production by transduced T cells.

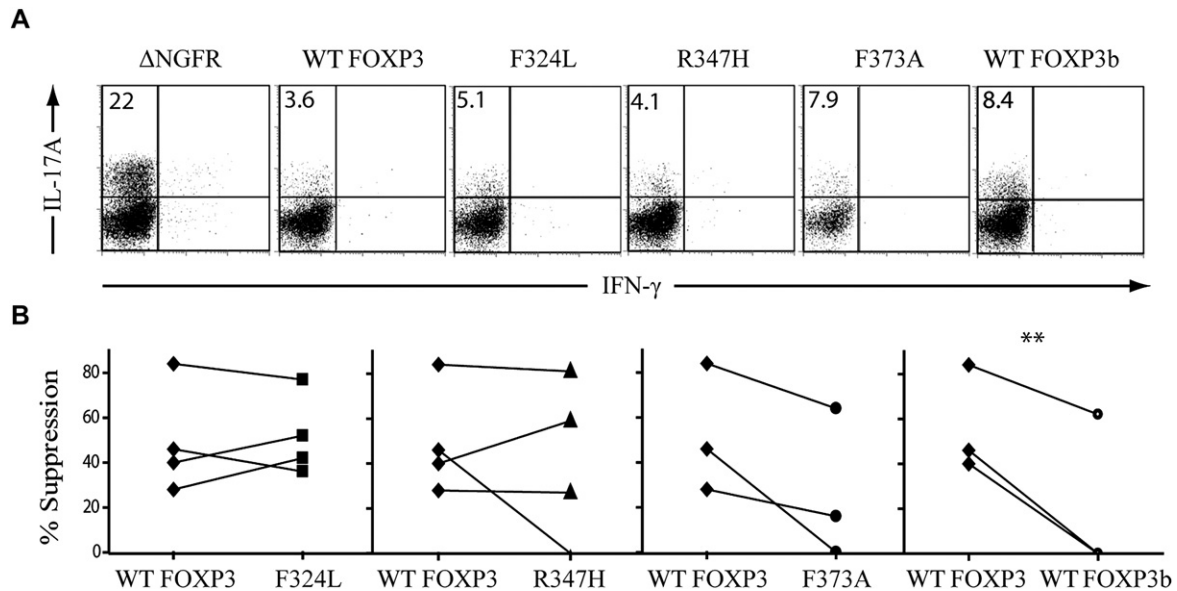


FIG 5. Expression of IL-17 and IFN- γ in T_H17 cells expressing WT, WT FOXP3, or IPEX mutant forms of FOXP3. **A**, Representative experiment. **B**, Summary of percent suppression of IL-17 production on the basis of IL-17 production by control transduced cells. Lines connect individual experiments, comparing percent suppression by WT FOXP3 to suppression by each of the IPEX mutants and to WT FOXP3b. Asterisks indicate a significant difference compared with WT FOXP3. *** $P < .001$; ** $P = .001-.01$; * $P = .01-.05$.

FOXP3 had only $3.0\% \pm 3.1\%$ demethylation of the TSDR ($n = 4$; data not shown) compared with nearly 100% demethylation in natural Treg cells.³⁹

Currently, the only curative treatment for IPEX is bone marrow transplantation.⁵ A recent report on chimerism after bone marrow transplant of a patient with IPEX showed that after 6 years, whereas the majority of $CD4^+$ Tconv cells were of recipient origin, donor chimerism in the $CD4^+CD25^{hi}$ T-cell subset was 53% to 91%.⁵⁹ Importantly, this patient has a null mutation that results in a complete lack of FOXP3 protein expression. This patient is in remission, suggesting that in the absence of FOXP3, defective Treg cells may be the major cause of disease. Nevertheless, because point mutants of FOXP3, which are expressed in patients with IPEX, were not completely defective at inducing a Treg phenotype, this protein likely has additional functions beyond its well characterized role in Treg cells. For example, FOXP3 may regulate Tconv cells or be required for Treg cell functions other than suppression of T cells such as inhibition of APCs or B cells.⁶⁰⁻⁶³

In conclusion, depending on the mutation, the full clinical manifestations of IPEX may not be exclusively caused by defects in Treg cells. Although both F373A and R347H are mutations located in the forkhead domain that cause severe IPEX, only F373A was significantly, but not completely, impaired in reprogramming Tconv cells into Treg cells. Furthermore, F324L, which causes a mild form of IPEX, did not display any significant defect in the best characterized cellular and molecular functions of FOXP3. The hypothesis that IPEX is not caused by a numerical deficit in Treg cells is supported by a recent study⁶⁴ in *Scurfy* mice finding that *Foxp3*-deficient Treg cells share many characteristics with WT Treg cells including *in vitro* anergy, decreased cytokine production, and gene expression profiles. Therefore, it seems likely that the immune deficiencies in patients with IPEX can be caused by changes in the function of FOXP3 outside

its currently recognized role in regulation of Treg cell development and function. Future investigation into the role of activation-induced FOXP3 in Tconv cells and how functions may be disrupted in IPEX will be essential to understand better the pathophysiology of this disease.

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Key messages

- Two point mutant forms of FOXP3 (R347H and F373A) that cause severe IPEX were not completely defective in reprogramming T cells into Treg cells.
- A point mutant form of FOXP3 (F324L) that causes mild IPEX was as effective as WT FOXP3 in reprogramming T cells into Treg cells.
- Factors other than functional defects in Treg cells likely contribute to the pathophysiology of IPEX.

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