# 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;126:1242-51.)**

***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 kd[20]; Eos[25]; retinoic acid receptor-related orphan receptor γ (ROR-γt)[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-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]

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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

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 gluccocorticoid-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 ([$^3$H]-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 [$^3$H]-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 [$^3$H]-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-17$^-$IFN-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 (***$P < .001$; **$P = .001$-$.01$; *$P = .01$-$.05$).