Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development

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Forkhead winged-helix transcription factor Foxp3 serves as the dedicated mediator of the genetic program governing CD25$^+$CD4$^+$ regulatory T cell (T_R) development and function in mice. In humans, its role in mediating T_R development has been controversial. Furthermore, the fate of T_R precursors in FOXP3 deficiency has yet to be described. Making use of flow cytometric detection of human FOXP3, we have addressed the relationship between FOXP3 expression and human T_R development. Unlike murine Foxp3$^-$ T cells, a small subset of human CD4$^+$ and CD8$^+$ T cells transiently upregulated FOXP3 upon *in vitro* stimulation. Induced FOXP3, however, did not alter cell-surface phenotype or suppress T helper 1 cytokine expression. Furthermore, only *ex vivo* FOXP3$^+$ T_R cells persisted after prolonged culture, suggesting that induced FOXP3 did not activate a T_R developmental program in a significant number of cells. FOXP3 flow cytometry was also used to further characterize several patients exhibiting symptoms of immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) with or without *FOXP3* mutations. Most patients lacked FOXP3-expressing cells, further solidifying the association between FOXP3 deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Interestingly, one patient bearing a *FOXP3* mutation enabling expression of stable FOXP3^{mut} protein exhibited FOXP3^{mut}-expressing cells among a subset of highly activated CD4$^+$ T cells. This observation raises the possibility that the severe autoimmunity in FOXP3 deficiency can be attributed, in part, to aggressive T helper cells that have developed from T_R precursors.

A significant body of evidence has been derived from rodent models demonstrating that, through Foxp3 expression, CD25$^+$CD4$^+$ regulatory T cells (T_R) develop as a separate lineage of CD4$^+$ T cells with a unique and vital function (1–3). T_R have also been identified in humans and have been shown to possess many of the same phenotypic and functional properties as their murine counterparts (4). Mutations of FOXP3 in humans lead to an early-onset, multisystem autoimmune syndrome known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) (5–7). *Foxp3^{null}* and *scurfy* mice exhibit an analogous autoimmune pathology (8, 9), suggesting that a similar function is served by FOXP3 across phylogeny.

Although it is well established that both murine and human T_R develop as a subset of CD4 single-positive thymocytes (10, 11), the conditions under which T_R arise in peripheral organs is less understood. In mice, no measurable role for Foxp3 has been found in the differentiation or function of non-T_R in response to T cell receptor (TCR) agonists (9). In contrast, human CD25$^-$CD4$^+$ and CD8$^+$ T cells have been shown to increase FOXP3 mRNA and protein levels upon activation, suggesting a cell-intrinsic role for FOXP3 in the regulation of T cell responses in humans (12–14). Furthermore, the existence of IPEX-like individuals that are phenotypically similar to IPEX but lack mutations within the coding region of the *FOXP3* gene calls into question the role of FOXP3 as the "master regulator" of human T_R development and function. Thus, two nonmutually exclusive models can be proposed for the role of FOXP3 in regulating immune responses in humans. In the first model, preexisting FOXP3$^+$ T_R are recruited to sites of active immune response where they suppress antigen-specific effector T cells and expand to control the intensity of the response. In the second model, FOXP3$^-$CD4$^+$ T cells responding to neoantigens expressed by target organs or to pathogens give rise to a clonal population consisting of both effector T cells and FOXP3$^+$ T_R, the latter of which may either exist transiently or give rise to long-lived T_R.

Whether the mechanisms of T_R development and function differ in humans and mice is currently an area of significant debate. Recent evidence suggests that the latter model of peripheral T_R development may be more operative in humans than in mice, because some groups have found that, unlike murine cells, stimulation of human CD25$^-$CD4$^+$ T cells results in considerable FOXP3 expression and development of suppressor activity (12, 15). Others have not observed such conversion of naïve T cells into FOXP3-expressing T_R *in vitro* (16). Determining whether humans generate large numbers of "adaptive" T_R during immune responses, and the mechanisms driving such T_R development, is of substantial basic and practical significance. To address these possibilities and to further examine the relationship between FOXP3 deficiency and IPEX, we investigated FOXP3 expression in *ex vivo* isolated and activated T cells from normal donors and IPEX patients using our recently developed flow cytometric methodology. Serendipitously, the identification in one patient of activated T cells expressing a loss-of-function mutant FOXP3 suggests the possibility that the severity of IPEX/*scurfy* autoimmunity may result from an alternative proinflammatory fate of T_R precursors.

Results and Discussion

Flow Cytometric Characterization of Human FOXP3$^+$ Cells. To examine the regulation of FOXP3 expression in individual human T cells, we developed methods for flow cytometric detection of FOXP3 using a novel mouse mAb (3G3) or a digoxigenin-conjugated rabbit polyclonal antibody. Both antibodies detect murine as well as human FOXP3, and their utility for single-cell detection of Foxp3 expression was demonstrated by using normal and *Foxp3^{null}* mice. Staining of mouse lymph node cells with either antibody revealed Foxp3 expression in the majority of CD25$^+$CD4$^+$ T cells and a small subset of CD25$^-$CD4$^+$ cells (Fig. 1 *A* and *B*). This Foxp3

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Abbreviations: Th, T helper; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; T_R, regulatory T cell; PBMC, peripheral blood mononuclear cell; TCR, T cell receptor; PE, phycoerythrin; PW, Perm/Wash.

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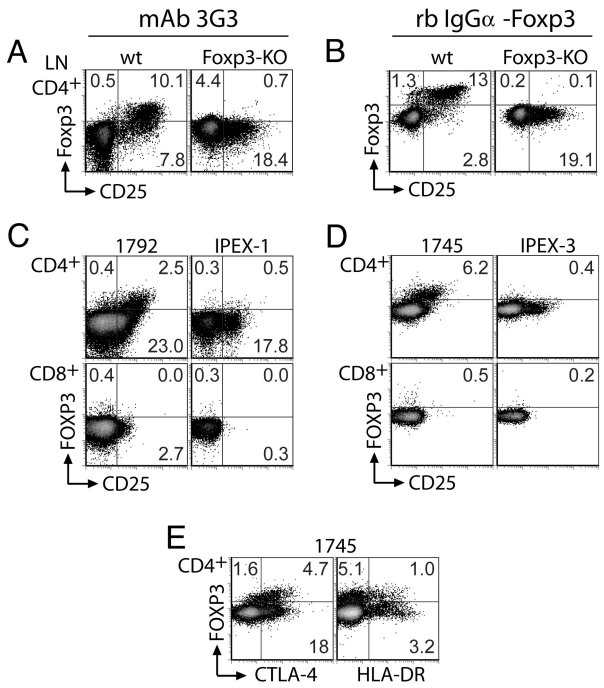

Fig. 1. Flow cytometric detection of Foxp3 in murine and human cells. (*A* and *B*) Normal or Foxp3-deficient mouse lymph node cells were stained for Foxp3 and cell-surface markers by using digoxigenin-conjugated mAb 3G3 (*A*) or Foxp3-specific rabbit antibody (*B*). $CD4^+$ gated lymphocytes are shown. (*C–E*) Normal (1792 and 1745) or FOXP3-deficient (IPEX) PBMC were stained for FOXP3 and lymphocyte markers by using digoxigenin-conjugated mAb 3G3 (*C*) or digoxigenin-conjugated Foxp3-specific rabbit antibody (*D* and *E*). Both $CD4^+$ and $CD8^+$ gated lymphocytes are shown. Additional IPEX-1 PBMC were not available for subsequent analysis with rabbit antibody. High background staining of $Foxp3^-$ cells is a consequence of the three-step staining procedure.

expression pattern was similar to that of *Foxp3*^{GFP} knockin mice (17). Reactivity with Foxp3 was specific, because no staining was observed with either antibody in *Foxp3*^{null} cells (Fig. 1 *A* and *B*). Specificity was further confirmed by mapping of the mAb 3G3 epitope to the amino-terminal portion of FOXP3, a domain unique among all forkhead-family transcription factors (Fig. 5, which is published as supporting information on the PNAS web site). No specific staining was observed in murine $CD8^+$ cells or non-T cells (data not shown).

FOXP3 expression profiles in human peripheral blood mononuclear cells (PBMC) were very similar to those observed in murine cells. All $CD25^{high}CD4^+$ cells, previously shown to exhibit potent suppressor function (4), were $FOXP3^+$, whereas only a minority of $CD25^{low}CD4^+$ and $CD25^-CD4^+$ cells exhibited FOXP3 expression. This finding is consistent with the observation that $CD25^{low}$ cells are not suppressive (18) (Fig. 1 *C* and *D*). Previous estimates have proposed that the human T_R subset constitutes ≈1–3% of $CD4^+$ T cells. However, the percentage of $FOXP3^+$ cells was found to be closer to 6% in normal donors using our FOXP3-specific rabbit polyclonal antibody. This finding is in complete agreement with recently described flow cytometric detection of human FOXP3 using another novel mAb (14). Similar to *Foxp3*^{null} mice, patients with *FOXP3* mutations affecting mRNA splicing (IPEX-1 and IPEX-3) have no detectable $FOXP3^+$ cells (Fig. 1 *C* and *D* and Table 1). Interestingly, $CD4^+$ cells from IPEX patients exhibited a similar proportion of $CD25^+$ cells as normal subjects, suggesting the presence of activated effector T helper (Th) cells despite the administration of immunosuppressants (Fig. 1 *C* and *D* and Table 1). $FOXP3^+CD4^+$ cells were also enriched in expression of the T cell activation markers CTLA-4 and HLA-DR. In contrast to the correlation seen between high CD25 expression and FOXP3 positivity, however, comparably high expression levels of CTLA-4 and HLA-DR were present on both $FOXP3^+$ and $FOXP3^-$ $CD4^+$ T cells (Fig. 1*E*). In the $CD8^+$ T cell compartment, there were negligible numbers of $FOXP3^+$ cells (compare with the IPEX sample that lacks FOXP3 expression altogether), showing that, in quiescent PBMC, FOXP3-expressing $CD8^+$ cells are rare (Fig. 1 *C* and *D*). For reasons likely due to variable epitope accessibility, our 3G3 mAb was somewhat less efficient than the rabbit polyclonal antibody at detecting FOXP3-expressing cells (Fig. 1). However, its utility and specificity for staining FOXP3 in humans is demonstrated here in normal and IPEX patient samples (Fig. 1).

FOXP3 Expression Is Induced Transiently in Some Human Non-T_R $CD4^+$ and $CD8^+$ T Cells upon Activation but Persists only in *in Vivo*-Generated T_R Cells. To investigate the degree to which *de novo* FOXP3 expression might occur in individual human T cells, we examined FOXP3 expression after TCR stimulation. Total or CD25-depleted PBMC were stimulated with varying doses of anti-CD3, and cells were analyzed by flow cytometry on days 3, 7, and 10 of culture. This regimen relies on "presentation" of anti-CD3 antibody to T cells by Fc receptors on antigen-presenting cells, a situation that we feel more closely resembles TCR activation in

Table 1. IPEX patients

Patient	Mutation	Dermatitis	Endocrinopathy type (age at onset)	Other*	Age and treatment when PBMC drawn
IPEX-1	210-210 + 1, GG > AC, splicing Δ	Eczema	IDDM (2 months)	AIHA/ITP ↑ IgE	5 months, FK506/steroids, TPN-dependent
IPEX-2-P1	c.751_753, del GAG, p.ΔE251	Eczema	IDDM (6 months) and thyroiditis	AIHA ↑ IgE	6 years, intermittent steroids
IPEX-2-P2	c.751_753, del GAG, p.ΔE251	Eczema	IDDM (6 months) and thyroiditis	↑ IgE	9 years, FK506
IPEX-3	g.−6247_−4859 del, splicing Δ	Eczema	None	Food allergies ↑ IgE	4 years, FK506
IPEX-like-1	N/A	Eczema	IDDM (2 years) and thyroiditis (6 years)	Nephrotic syndrome	11 years, FK506
IPEX-like-2	N/A	Eczema	Thyroiditis	Candidiasis ↑ IgE	3 years, azathioprine
IPEX-like-3	N/A	Eczema and alopecia	IDDM (2 years)	None	3 years
IPEX-like-4	N/A	Exfoliative dermatitis and alopecia	None	Persistent AIHA ↑ IgE	4 months, CsA

Mutation nomenclature is according to ref. 28. IDDM, insulin-dependent diabetes mellitus; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia; TPN, total parenteral nutrition; N/A, not available; ↑, high concentration.
*All patients had moderate to severe enteropathy with profuse watery diarrhea.

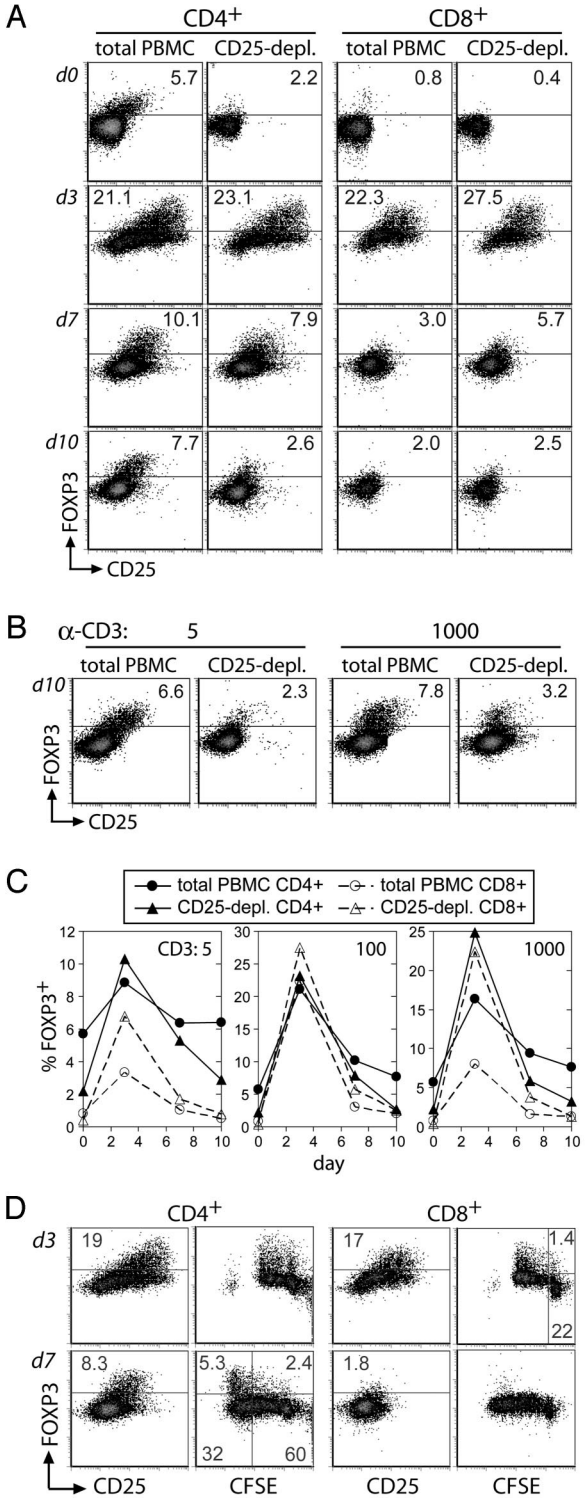

Fig. 2. Analysis of FOXP3 expression in activated human CD4$^+$ and CD8$^+$ T cells. (*A–C*) Total or CD25-depleted PBMC from donor 1745 were stimulated with 5, 100, or 1000 ng/ml anti-CD3. FOXP3 and CD25 expression on CD4$^+$ and CD8$^+$ cells were assessed at days 3, 7, and 10 of culture. Shown are expression profiles for 100 ng/ml anti-CD3 (*A*), 5 or 1,000 ng/ml anti-CD3 for CD4$^+$ gated cells (*B*), and the plotted percentage of gated cells expressing FOXP3 (*C*). FOXP3 was detected with digoxigenin-conjugated FOXP3-specific rabbit antibody. (*D*) Total PBMC from donor 1745 were labeled with CFSE and stimulated with 100 ng/ml anti-CD3. FOXP3 expression was assessed at days 3 and 7 with digoxigenin-conjugated rabbit antibody. Data are representative of four separate experiments and three normal adult donors.

response to its natural ligands (i.e., peptide/MHC complexes) than plate- or bead-immobilized antibodies. A dramatic increase in the percentage of FOXP3$^+$ cells among both CD4$^+$ and CD8$^+$ T cells was observed after stimulation, with up to 25% of CD4$^+$ cells and 27% of CD8$^+$ cells expressing FOXP3 on day 3 (Fig. 2 *A* and *C*). The proportion of FOXP3$^+$ T cells diminished progressively over time to near baseline levels by day 10. Interestingly, the relative loss of FOXP3 expression was most dramatic for cell populations that contained fewer FOXP3$^+$ cells before activation (CD8$^+$ cells and CD4$^+$ cells from CD25-depleted PBMC). In contrast, CD4$^+$ cells in cultures of total PBMC retained a FOXP3$^+$CD25$^+$CD4$^+$ subpopulation on day 10 of culture that was strikingly similar to freshly isolated PBMC (Fig. 2 *A* and *B*). This pattern of transient FOXP3 expression was observed in cells from four unrelated normal donors and was consistent among monoclonal 3G3, rabbit polyclonal antibody, or monoclonal 259D (14) (data not shown). Furthermore, T cell costimulation was required for FOXP3 induction, because activation of purified T cells with plate-bound anti-CD3 and anti-CD28, but not anti-CD3 alone, promoted similar transient FOXP3 expression (Fig. 6, which is published as supporting information on the PNAS web site).

The substantial size of the FOXP3$^+$ cell population after T cell activation suggests that many of these cells may arise by transient, activation-induced, *de novo* expression of FOXP3 in non-T_R. However, this is difficult to ascertain because of a preexisting population of FOXP3$^+$CD25$^-$CD4$^+$ T cells potentially capable of *in vitro* expansion. Indeed, similar experiments with mouse cells revealed a striking enrichment of Foxp3$^+$ cells because of selective outgrowth (Fig. 7, which is published as supporting information on the PNAS web site). To further examine human FOXP3 induction *in vitro*, PBMC were CFSE-labeled before stimulation with anti-CD3. Cells were evaluated for proliferative responses and FOXP3 expression levels by flow cytometry. On day 3, when increased numbers of FOXP3$^+$ cells were readily observed, FOXP3 expression on both CD4$^+$ and CD8$^+$ cells was not confined to highly divided CFSE^{low} cells (Fig. 3*D*). Specifically, FOXP3 was found to be expressed in ≈6% of CD8$^+$ T cells that had not yet undergone cell division (Fig. 2*D*). Thus, unlike murine cells, some human CD8$^+$ and likely CD4$^+$ T cells are capable of *de novo* FOXP3 induction *in vitro*. Although FOXP3$^+$ cells on day 7 exhibited a high degree of CFSE dilution, it is likely that most of these cells derived from the efficient proliferation of preexisting T_R because the depletion of CD25$^+$ cells from starting cultures (while not dramatically affecting the degree of FOXP3 induction on day 3) results in a paucity of FOXP3$^+$ cells at later time points (Fig. 2 *A–C*). Importantly, unlike up-regulation of CD25, only a subset of T cells induced FOXP3 expression, suggesting that FOXP3 induction is stochastic or that some peripheral T cells are poised, i.e., precommitted, to express FOXP3.

Induced FOXP3 Does Not Suppress Th1 Cytokine Synthesis. Next we sought to determine whether the induction of FOXP3 resulted in a T_R-like phenotype. Induced FOXP3 did not correlate with altered CD25, glucocorticoid-induced TNF receptor, or CD27 expression (Fig. 2*A* and data not shown); thus, it was not possible to isolate cells expressing induced FOXP3 for direct suppressor function studies. Ectopic expression of high levels of FOXP3 in naïve human CD4$^+$ T cells suppresses IL-2 and IFN-γ production (16, 19), mirroring the inability of naturally developing T_R to produce these cytokines. Thus, analysis of intracellular cytokine production should serve as an indirect way to assess acquisition of some T_R properties by activated T cells.

To determine whether the induced FOXP3 suppressed these cytokines, cultured cells were examined for FOXP3, IL-2, and IFN-γ expression. As expected, the FOXP3$^+$CD4$^+$ T_R in freshly isolated PBMC did not express either IL-2 or IFN-γ in response to activation on day 0 (Fig. 3). On days 3 and 7 after stimulation, both cytokines were expressed by FOXP3$^-$CD4$^+$ and FOXP3^{low}CD4$^+$

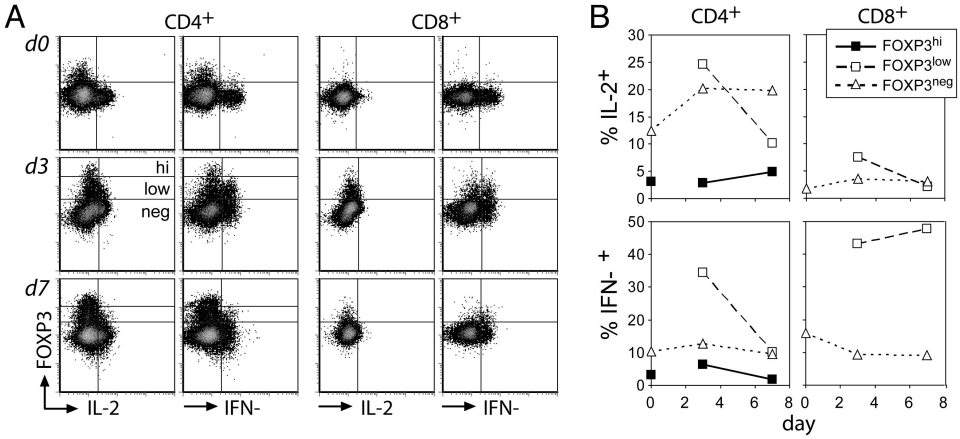

Fig. 3. Induced FOXP3 does not suppress IL-2 or IFN-γ synthesis. (*A*) Freshly isolated or stimulated (100 ng/ml anti-CD3) total PBMC from donor 1745 were incubated with PMA, ionomycin, and monensin and stained for FOXP3 (rabbit IgG-digoxigenin), IL-2, IFN-γ, and surface markers as described in *Materials and Methods*. (*B*) The percentage of cytokine-expressing cells among FOXP3^{high}, FOXP3^{low}, or FOXP3^{-} cells is plotted. The distinction between high and low FOXP3 expression was not made for CD4^{+} cells and CD8^{+} cells on day 0. Data are representative of three separate experiments.

cells but not by a distinguishable FOXP3^{high} population observed among CD4^{+} but not CD8^{+} T cells. The notable lack of Th1 cytokine expression by FOXP3^{high}CD4^{+} cells suggests that high levels of FOXP3 are required to suppress cytokine synthesis or that this population was derived from preexisting FOXP3^{+}CD4^{+} T$_R$ present in the starting population (Fig. 3). The latter hypothesis is supported by the observation that the FOXP3^{high}CD4^{+} population was 20% less (day 3) and 75% less (day 7) abundant in the CD25-depleted vs. undepleted PBMC cultures (data not shown). FOXP3^{+}CD8^{+} cells demonstrated an expected lack of IL-2 production but efficiently expressed IFN-γ on both day 3 and day 7, indicating that FOXP3 also did not promote a T$_R$-like transcriptional program in CD8^{+} T cells. Similar results were obtained with another FOXP3-specific monoclonal 259D using PBMC from a second donor and costaining for TNF-α in addition to IL-2 and IFN-γ (Fig. 8, which is published as supporting information on the PNAS web site). Cells with induced FOXP3 are likely to downregulate its expression rather than undergo apoptosis, because costaining with the caspase active-site reporter substrate FITC-VAD-FMK identified subsets of apoptotic FOXP3^{-}CD4^{+} but not FOXP3^{+}CD4^{+} cells at days 3 and 7 (data not shown). Together, these findings suggest that FOXP3 induced *in vitro* upon activation of human non-T$_R$ does not promote T$_R$ phenotype development, whereas FOXP3^{+} T$_R$ generated *in vivo* persist and maintain their functional characteristics after *in vitro* expansion.

FOXP3 Expression in IPEX Syndrome. Although *FOXP3* mutations have been characterized in more than two-thirds of IPEX patients, we have identified a subgroup of patients exhibiting a similar pattern of autoimmune characteristics but lacking *FOXP3* coding or splice-site mutations. In such individuals, identified as IPEX-like (Table 1), FOXP3 deficiency may result from uncharacterized *FOXP3* promoter mutations or from mutations in genes required for FOXP3 expression. Alternatively, FOXP3 expression may be intact, and the disease may result from mutations in other genes affecting T cell regulation. To better characterize the etiology of the autoimmune pathology in these IPEX-like patients, PBMC were analyzed for FOXP3 expression with mAb 3G3. Of four IPEX-like patients, three (IPEX-like-1, -2, and -4) lacked FOXP3 expression in the CD25^{high}CD4^{+} cell population, whereas IPEX-like-3 exhibited moderate FOXP3 expression in CD25^{high}CD4^{+} cells (Fig. 4*A*). Thus, we have linked three of four IPEX-like patients who lack FOXP3 coding mutations with FOXP3 deficiency. Promoter mutations that completely or partially attenuate FOXP3 expression are the most likely cause for FOXP3 deficiency in these individuals, the identification of which will significantly advance our understanding of the factors and signals that promote *FOXP3* transcription.

Among the three IPEX patients, two (IPEX-1 and IPEX-3) have mutations in the 5′ portion of the gene that lead to aberrant mRNA splicing and absence of protein expression. A third IPEX patient (IPEX-2) harboring a single, in-frame amino acid deletion (ΔE251) within the leucine zipper of FOXP3 was also identified. This mutation was of particular interest because it should allow for expression of a full-length, mutant FOXP3 protein. Indeed, ectopic expression of native or mutant FOXP3 in both human fibroblasts and primary CD4^{+} T cells established that FOXP3$^{\Delta E251}$ protein was stable and could be efficiently detected by flow cytometry (Fig. 9, which is published as supporting information on the PNAS web site). Furthermore, FOXP3$^{\Delta E251}$ was unable to dimerize or to suppress transcription from an IL-2 promoter–luciferase reporter construct, confirming a lack of functional activity (T.R.T., unpublished observations). The presence of the classic IPEX phenotype in this patient strongly argues against FOXP3$^{\Delta E251}$ promoting significant T$_R$ activity. Thus, FOXP3$^{\Delta E251}$ protein should serve as a natural reporter to examine FOXP3 expression in the apparent absence of FOXP3 function, thereby advancing our understanding of the requirements for persistence of T$_R$ precursors as well as the nature of autoimmune effector cells in IPEX. Specifically, the presence of FOXP3$^{\Delta E251+}$ cells in IPEX-2 PBMC would indicate that cells receiving signals that promote FOXP3 expression are able to survive in the absence of FOXP3 function. Such cells could represent those that either (*i*) attempted T$_R$ development during thymic maturation and migrated to the periphery or (*ii*) induced FOXP3$^{\Delta E251}$ expression in peripheral tissues, perhaps in response to autoantigens. Alternatively, the lack of a FOXP3$^{\Delta E251}$ population would indicate that FOXP3 function is required for the survival of cells committed to the T$_R$ differentiation pathway.

Two IPEX-2 PBMC samples (P1 and P2) were obtained 3 years apart. The first sample (IPEX-2-P1) was drawn after treatment only with intermittent corticosteroid therapy, before the initiation of other potent immunosuppressants. The second (IPEX-2-P2) was drawn after 2 years of treatment with FK506. Analysis of FOXP3$^{\Delta E251}$ expression in each of these samples revealed intriguing differences. IPEX-2-P1 contained a population of large CD4^{+} cells expressing very high levels of CD25 (designated CD25^{++}) (Fig. 4 *A* and *B* and data not shown). Thirty-three percent of these cells expressed FOXP3$^{\Delta E251}$, but the presence of aggressive systemic autoimmune disease in the patient at the time that the sample was drawn argues against these cells having any significant regulatory function. In contrast, IPEX-2-P2 lacked this population of large

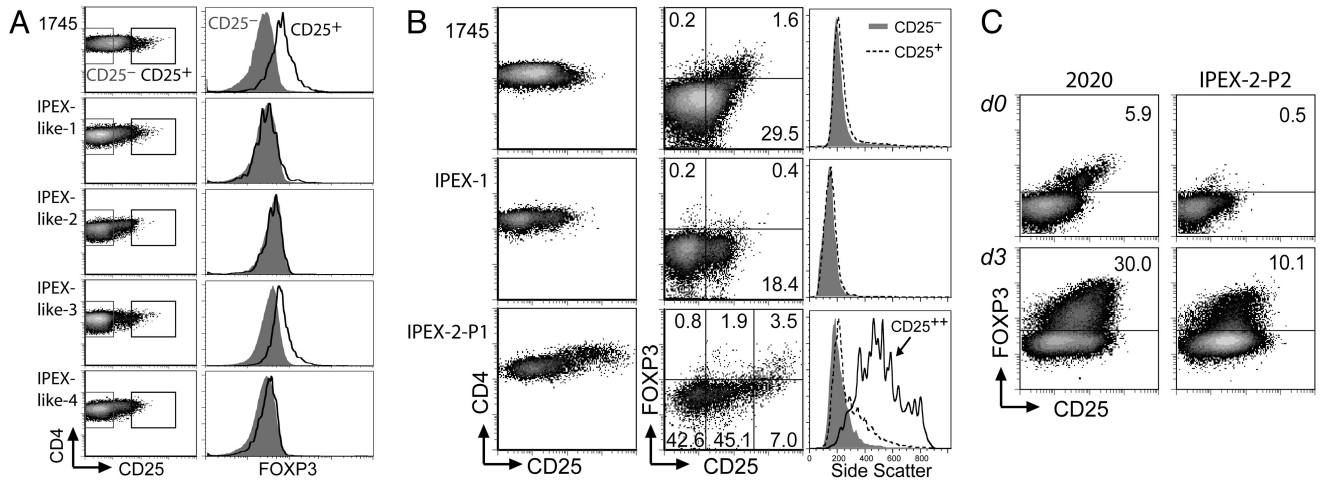

Fig. 4. FOXP3 expression in IPEX. (*A* and *B*) Normal (1745), IPEX-like (*A*), and IPEX (*B*) PBMC were stained for cell-surface markers and FOXP3 with mAb 3G3. Gated $CD4^+$ cells are shown. Histograms show FOXP3 expression (*A*) and side scatter (*B*) on $CD4^+$ cells expressing varying degrees of CD25 as delineated in the adjacent 2D plots. Because the staining procedure results in a decrease in forward scatter, side scatter is a better indicator of cell size. Staining was performed before the development of protocols by using digoxigenin-conjugated rabbit antibody, but additional PBMC from these patients were not available for further study. PBMC shown in *A* and *B* were stained in separate experiments. (*C*) Freshly isolated or simulated (100 ng/ml anti-CD3; 3 days) normal (2020) or IPEX-2-P2 PBMC were stained for FOXP3 with mAb 259D (14).

$CD25^{++}CD4^+$ cells and possessed a greatly reduced percentage of cells expressing $FOXP3^{\Delta E251}$ (Fig. 4*C* and data not shown). Despite the paucity of $FOXP3^{\Delta E251+}$ cells in freshly isolated PBMCs, $FOXP3^{\Delta E251}$ expression was induced in 10% of $CD4^+$ IPEX-2-P2 PBMC upon stimulation with anti-CD3 for 3 days, mirroring the kinetics of induction observed in control PBMC. We hypothesize that the large $CD25^{++}CD4^+$ cells found in IPEX-2-P1 are likely to represent aggressive autoreactive effector T cells, some of which also expressed $FOXP3^{\Delta E251}$, and that potent T cell-directed immunosuppression with FK506 resulted in the loss of this population.

In the context of our findings *in vitro*, two nonmutually exclusive potential mechanisms may explain the presence of $FOXP3^{\Delta E251}$-expressing $CD25^{++}CD4^+$ T cells in IPEX-2-P1. First, T_R precursors that did not receive appropriate signals to continue down a T_R developmental pathway because of lack of functional FOXP3 may have persisted as $FOXP3^{\Delta E251}$-expressing autoreactive effector T cells (i.e., cells bearing TCRs that normally promote thymic T_R development). Alternately, such cells may have arisen from effector T cells that have induced $FOXP3^{\Delta E251}$ expression in response to activation (i.e., cells normally suppressed by T_R). If the $FOXP3^{\Delta E251+}CD4^+$ cells arose from non-T_R precursors under conditions similar to those that promote FOXP3 induction *in vitro*, then a similar population may exist among $CD8^+$ IPEX-2-P1 cells because we have observed FOXP3 induction with equal efficiency in both $CD4^+$ and $CD8^+$ T cells. IPEX-2-P1 $CD8^+$ cells contained a $CD25^{++}$ subset similar to their $CD4^+$ counterparts, suggesting that some $CD8^+$ T cells were also highly reactive to self antigens (Fig. 10, which is published as supporting information on the PNAS web site); however, the high degree of $FOXP3^{\Delta E251}$ expression found in $CD25^{++}CD4^+$ cells was not observed (Figs. 4*B* and 10). Thus, signals unique to $CD4^+$ cells appear to promote *FOXP3* transcription in FOXP3 deficiency. If FOXP3 does not normally rescue T_R precursors from thymic negative selection, such a signal may derive from the increased TCR affinity T_R typically display for self-peptide/MHC ligands (20, 21). Our recent findings of T_R-specific TCRs expressed in activated $CD25^+CD4^+$ T cells from *Foxp3*^{null} mice support this hypothesis (22).

In conclusion, we have presented the first flow cytometric analysis of human FOXP3 expression in activated human PBMC, demonstrating that FOXP3 induction can be uncoupled from T_R development. Although some $FOXP3^-$ T cells up-regulated FOXP3 upon *in vitro* activation, Th1 cytokine synthesis was not blocked. Furthermore, under conditions that favored the persistence of *in vivo*-generated T_R, long-lived T_R were not readily derived from activated cells. *In vivo*, the identification of $FOXP3^{\Delta E251+}CD25^{++}CD4^+$ T cells in IPEX-2-P1 suggests that either similar induction can occur *in vivo* or autoreactive progeny of T_R precursors contribute significantly to the severity of IPEX symptomology. Although these two possibilities are not mutually exclusive, the latter scenario is attractive in that it associates self-reactive TCRs, i.e., those that promote T_R development, with T cells responsible for the multiorgan pathology observed in FOXP3-deficient humans and mice.

Although our findings reveal a lack of functional consequences of transiently induced FOXP3, others have reported *de novo* generation of $FOXP3^+$ suppressive T_R in more long-term cultures (12, 15). Our findings support the possibility that preexisting T_R, capable of efficient expansion *in vitro* when in the presence of IL-2-producing T cells, may contribute to the generated T_R population in these experimental systems. Because we have observed a correlation between high FOXP3 expression and repression of Th1 cytokines, sustained expression of high levels of FOXP3 may be required to promote T_R development *in vitro*. Indeed, our group and others have observed that ectopic expression of only high levels of murine or human FOXP3 results in the acquisition of T_R phenotype and function (J. Fontenot, personal communication) (23). Although our methods for T cell activation did not result in sustained, high-level expression of induced FOXP3, we cannot exclude the possibility that some experimental conditions may promote such expression and subsequent T_R development.

In normal individuals, acute T cell stimulation by high-affinity ligands can occur in response to various forms of neoantigen, including infectious agents, vaccines, alloantigens presented after organ transplantation, and self-antigens in the setting of graft-versus-host disease. Should FOXP3 induction occur in such highly activated T cells, as we have observed *in vitro*, the degree and longevity of its expression and consequential T_R development is likely to be effected by the maturation state of antigen-presenting dendritic cells (24–26). In mice, similar transient *de novo* Foxp3 expression has recently been reported for highly activated T cells stimulated *in vivo* by dendritic cells presenting foreign antigen, whereas only low levels of antigen in the absence of proinflammatory signals resulted in *de novo* T_R development (27). Our findings support the distinct possibility that transient up-regulation of

FOXP3 under proinflammatory conditions may not promote immunosuppressive function in contrast to that mediated by preexisting T_R responding to the same antigens. In aggregate, our data suggest that, despite the capacity for FOXP3 induction after TCR ligation, human T cells require sustained high-level FOXP3 expression for the acquisition of T_R function. Although such conditions may exist for T_R precursors in IPEX, they are not sufficient to elicit suppressor function in the absence of functional FOXP3.

Materials and Methods

Antibodies. Rabbits and mice were immunized with bacterially expressed recombinant His-tagged full-length murine Foxp3 (gift of Fred Ramsdell; Celltech R&D, Bothell, WA) purified on Ni-NTA-Agarose (Qiagen, Venlo, The Netherlands). Polyclonal antibodies were produced by immunizing rabbits (R&R Rabbitry, Stanwood, WA) every 21 days with 250 μg of His-Foxp3. Hybridoma 3G3 was generated by priming mice with 75 μg of His-Foxp3 followed by three 30-μg boosts before fusion and clone screening by ELISA. Positive clones were subcloned and expanded in GIBCO Hybridoma-SFM. Anti-Foxp3 antibodies were isolated from rabbit antisera or hybridoma supernatant with protein A or protein G Sepharose affinity chromatography (Amersham Pharmacia Biosciences). Antibodies were labeled with digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (Roche Diagnostics, Indianapolis).

PBMC Donors. Normal human PBMC were obtained from volunteer donors by leukopheresis. Participants gave informed consent per guidelines of the Institutional Review Board of the Fred Hutchinson Cancer Research Center. IPEX PBMC were isolated from venous blood for the molecular diagnosis of IPEX syndrome by sequence analysis and flow cytometry after consent of the patients.

T Cell Stimulation. Total or CD25-depleted (MACS, Miltenyi Biotec) pooled mouse lymph node and spleen cells or human PBMC were cultured at 4×10^6 cells per well (24-well plates) with titrated anti-CD3 (2C11.145 or OKT3) in mouse cell medium (DMEM/10% FBS/50 μM 2-mercaptoethanol/10 mM Hepes/2 mM L-glutamine/1 mM sodium pyruvate/penicillin–streptomycin) or human cell medium (RPMI medium 1640/10% human serum/50 μM 2-mercaptoethanol/12.5 mM Hepes/6 mM L-glutamine/23.8 mM sodium bicarbonate/penicillin–streptomycin).

Flow Cytometry. For staining with Foxp3-specific rabbit polyclonal IgG, cells were fixed in Cytofix/Cytoperm (BD Biosciences) for 30 min on ice, washed once in DMEM/5% FBS, and frozen at −80°C in DMEM/20% FBS/10% DMSO. Cells were thawed, washed twice in Perm/Wash (PW) (BD Biosciences), and refixed in Cytofix/Cytoperm for 4 min on ice. Cells were washed once with cold DMEM/5% FCS and twice with PBS, resuspended in PBS/500 μg/ml DNase (Roche, Indianapolis)/4 mM $MgCl_2$, and incubated at room temperature for 30–40 min (mouse cells) or 10 min (human cells). Cells were then stained in PW supplemented with 350 mM NaCl (PW500) with either 200 μg/ml normal goat IgG (Jackson ImmunoResearch) (mouse cells) or 5% normal rabbit serum (Jackson ImmunoResearch) (human cells). After 5–10 min, anti-Foxp3 rabbit IgG or digoxigenin-labeled anti-Foxp3 rabbit IgG was added to 10 μg/ml. After three washes in PW500, cells were stained with either 10 μg/ml biotinylated goat anti-rabbit IgG with 200 μg/ml normal goat IgG (mouse cells) or 5 μg/ml biotinylated mouse anti-digoxin mAb with 5% normal mouse serum (human cells) (all Jackson ImmunoResearch reagents). After 20–30 min at room temperature, cells were washed three times with PW stained in PW with allophycocyanin-conjugated streptavidin (BD Biosciences) and other fluorophore-conjugated antibodies specific for cell-surface antigens. After a 20-min incubation at room temperature, cells were washed twice in PW, resuspended in PBS, and analyzed on a FACSCalibur or FACSCanto flow cytometer (BD Biosciences). For staining with digoxigenin-labeled Foxp3-specific mouse mAb (3G3-dig), mouse cells were incubated with 200 μg/ml DNase whereas human cells were neither refixed nor treated with DNase. Normal mouse serum (5%) (Jackson ImmunoResearch) was included during staining with both 3G3-dig and the secondary anti-digoxin reagent. In later experiments, FOXP3 was detected with Alexa Fluor 488-conjugated 259D (14) according to the manufacturer's protocols (BioLegend, San Diego).

For cytokine staining, cells were cultured with PMA (40 ng/ml), ionomycin (1 μg/ml), and monensin (3 μM) for 5 h (day 0 PBMC) or 3 h (cultured PBMC) before fixation and storage at −80°C. Cells were stained with IL-2-FITC, IFN-γ-phycoerythrin (PE), 5% normal mouse serum, and cell-surface markers during incubation with the anti-digoxigenin secondary reagent. Cells were costained with CD4-peridinin chlorophyll protein (SK3), CD8-FITC or CD8-allophycocyanin Cy7 (RPA-T8), CD25-PE or CD25-PECy7 (M-A251), CTLA-4-PE (BNI3), and HLA-DR-PE (G46-6) (BD Biosciences).

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