For the article “Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development,” by Marc A. Gavin, Troy R. Torgerson, Evan Houston, Paul deRoos, William Y. Ho, Asbjørg Stray-Pedersen, Elizabeth L. Ocheltree, Philip D. Greenberg, Hans D. Ochs, and Alexander Y. Rudensky, which appeared in issue 17, April 25, 2006, of Proc. Natl. Acad. Sci. USA (103, 6659–6664; first published April 14, 2006; 10.1073/pnas.0509484103), the authors note the incorrect placement of the α in Fig. 1B. In addition, the position of Fig. 1E was incorrect. The authors also note the omission of the γ after each instance of “IFN-” in Fig. 3. The corrected figures and their legends appear below. These errors do not alter the conclusions of the article.

**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<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> gated lymphocytes are shown. Additional IPEX-1 PBMC were not available for subsequent analysis with rabbit antibody. High background staining of Foxp3<sup>+</sup> cells is a consequence of the three-step staining procedure.

**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<sup>hi</sup>, FOXP3<sup>lo</sup>, or FOXP3<sup>−</sup> cells is plotted. The distinction between high and low FOXP3 expression was not made for CD4<sup>+</sup> cells and CD8<sup>+</sup> cells on day 0. Data are representative of three separate experiments.
PHYSIOLOGY. For the article “Hypoxia-inducible myoglobin expression in nonmuscle tissues,” by Jane Fraser, Luciane Vieira de Mello, Deborah Ward, Huw H. Rees, Daryl R. Williams, Yongchang Fang, Andrew Brass, Andrew Y. Gracey, and Andrew R. Cossins, which appeared in issue 8, February 21, 2006, of *Proc. Natl. Acad. Sci. USA* (103, 2977–2981; first published February 9, 2006; 10.1073/pnas.0508270103), the authors note that in the author line, the affiliations for Luciane Vieira de Mello, Yongchang Fang, and Andrew Brass appeared incorrectly, due to a printer’s error. In addition, the name Yongchang Fang should have appeared as Yongxiang Fang. The online version has been corrected. The corrected author and affiliation lines and the original author footnotes appear below.

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www.pnas.org/cgi/doi/10.1073/pnas.0602302103

GENETICS. For the article “Long-range multilocus haplotype phasing of the MHC,” by Zhen Guo, Leroy Hood, Mari Malkki, and Effie W. Petersdorf, which appeared in issue 18, May 2, 2006, of *Proc. Natl. Acad. Sci. USA* (103, 6964–6969; first published April 21, 2006; 10.1073/pnas.0602286103), the authors note that a grant was incorrectly cited. In line 5 of the Acknowledgments, “National Institutes of Health Grants AI18029” should have read “National Institutes of Health Grants CA18029.”

www.pnas.org/cgi/doi/10.1073/pnas.0603506103

GENETICS. For the article “Insights into TOR function and rapamycin response: Chemical genomic profiling by using a high-density cell array method,” by Michael W. Xie, Fulai Jin, Heejun Hwang, Seungmin Hwang, Vikram Anand, Mara C. Duncan, and Jing Huang, which appeared in issue 20, May 17, 2005, of *Proc. Natl. Acad. Sci. USA* (102, 7215–7220; first published May 9, 2005; 10.1073/pnas.0500297102), the authors wish to add a reference to a paper by C. W. Xu (1), who employed a microarrayer to fabricate bacterial and yeast cell microarrays on nitrocellulose membrane.


www.pnas.org/cgi/doi/10.1073/pnas.0603451103
Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development


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For the Regulation of Human CD25⁺CD4⁺ Regulatory T Cells

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). Foxp3mut 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⁺CD25⁺ T cells, 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).

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 Foxp3mut 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. 1A and B). This Foxp3

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

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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www.pnas.org/cgi/doi/10.1073/pnas.0509484103
FOXP3 expression profiles in human peripheral blood mononuclear cells (PBMC) were very similar to those observed in murine cells. All CD25highCD4+ cells, previously shown to exhibit potent suppressor function (4), were FOXP3+, whereas only a minority of CD25lowCD4+ and CD25−CD4+ cells exhibited FOXP3 expression. This finding is consistent with the observation that CD25low cells are not suppressive (18) (Fig. 1 C and D). Previous estimates have proposed that the human Tg 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 Foxp3null 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-Th CD4+ and CD8+ T Cells upon Activation but Persists only in in Vivo-Generated Th 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

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**Table 1. IPEX patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Dermatitis</th>
<th>Endocrinopathy type (age at onset)</th>
<th>Other*</th>
<th>Age and treatment when PBMC drawn</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPEX-1</td>
<td>210-210 + 1, GG &gt; AC, splicing 3</td>
<td>Eczema</td>
<td>IDDM (2 months)</td>
<td>AIHA/ITP</td>
<td>5 months, FK506/steroids, TPN-dependent</td>
</tr>
<tr>
<td>IPEX-2-P1</td>
<td>c.751,753, del GAG, p.E251</td>
<td>Eczema</td>
<td>IDDM (6 months) and thyroiditis</td>
<td>IgE</td>
<td>6 years, intermittent steroids</td>
</tr>
<tr>
<td>IPEX-2-P2</td>
<td>c.751,753, del GAG, p.E251</td>
<td>Eczema</td>
<td>IDDM (6 months) and thyroiditis</td>
<td>IgE</td>
<td>9 years, FK506</td>
</tr>
<tr>
<td>IPEX-3</td>
<td>g.6247–4859 del, splicing 3</td>
<td>Eczema</td>
<td>None</td>
<td>Food allergies</td>
<td>4 years, FK506</td>
</tr>
<tr>
<td>IPEX-like-1</td>
<td>N/A</td>
<td>Eczema</td>
<td>IDDM (2 years) and thyroiditis (6 years)</td>
<td>Nephrotic syndrome</td>
<td>11 years, FK506</td>
</tr>
<tr>
<td>IPEX-like-2</td>
<td>N/A</td>
<td>Eczema</td>
<td>Thyroiditis</td>
<td>Candidiasis</td>
<td>3 years, azathioprine</td>
</tr>
<tr>
<td>IPEX-like-3</td>
<td>N/A</td>
<td>Eczema and alopecia</td>
<td>IDDM (2 years)</td>
<td>None</td>
<td>3 years</td>
</tr>
<tr>
<td>IPEX-like-4</td>
<td>N/A</td>
<td>Exfoliative dermatitis and alopecia</td>
<td>None</td>
<td>Persistent AIHA</td>
<td>4 months, CsA</td>
</tr>
</tbody>
</table>

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.
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-TR. 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 CFSElow cells (Fig. 3D). Specifically, FOXP3 was found to be expressed in ∼6% of CD8− T cells that had not undergone cell division (Fig. 2D). 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 Tg 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 Tg-like phenotype. Induced FOXP3 did not correlate with altered CD25, glucocorticoid-induced TNF receptor, or CD27 expression (Fig. 2A 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 naive human CD4+ T cells suppresses IL-2 and IFN-γ production (16, 19), mirroring the inability of naturally developing Tg to produce these cytokines. This is likely that most of these cells derived from the efficient proliferation of preexisting Tg 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.
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 pathologies 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. 4A). 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^{ΔE251} 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^{ΔE251} 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 argue against FOXP3^{ΔE251} promoting significant T_R activity. Thus, FOXP3^{ΔE251} 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^{ΔE251} 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^{ΔE251} expression in peripheral tissues, perhaps in response to autoantigens. Alternatively, the lack of a FOXP3^{ΔE251} 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 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^{ΔE251} 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. 4A and B and data not shown). Thirty-three percent of these cells expressed FOXP3^{ΔE251}, 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
PBMC were stained for FOXP3 with mAb 259D (14). 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 FOXP3ΔE251+ (Fig. 4C and data not shown). Despite the paucity of FOXP3ΔE251+ cells in freshly isolated PBMCs, FOXP3ΔE251 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 FOXP3ΔE251+ 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 FOXP3ΔE251+ expressing CD25++ CD4+ T cells in IPEX-2-P1. First, TR precursors that did not receive appropriate signals to continue down a TR developmental pathway because of lack of functional FOXP3 may have persisted as FOXP3ΔE251+ expressing autoreactive effector T cells (i.e., cells bearing TCRs that normally promote thymic TR development). Alternately, such cells may have arisen from effector T cells that have induced FOXP3ΔE251 expression in response to activation (i.e., cells normally suppressed by TRs). If the FOXP3ΔE251+ CD4+ cells arose from non-TR 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 FOXP3ΔE251 expression found in CD25++ CD4+ cells was not observed (Figs. 1B and 10). Thus, signals unique to CD4+ cells appear to promote FOXP3 transcription in FOXP3 deficiency. If FOXP3 does not normally rescue TR precursors from thymic negative selection, such a signal may derive from the increased TCR affinity TR typically display for self-peptide/MHC ligands (20, 21). Our recent findings of FOXP3-specific TCRs expressed in activated CD25++ CD4+ T cells from Foxp3null 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 TR 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 TR, long-lived TR were not readily derived from activated cells. In vivo, the identification of FOXP3ΔE251+ CD25++ CD4+ T cells in IPEX-2-P1 suggests that either similar induction can occur in vivo or autoreactive progeny of TR 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 TR 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 TR in more long-term cultures (12, 15). Our findings support the possibility that preexisting TR, capable of efficient expansion in vitro when in the presence of IL-2-producing T cells, may contribute to the generated TR 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 TR 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 TR 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 TR 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 TR development is likely to be affected 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 vitro by dendritic cells presenting foreign antigen, whereas only low levels of antigen in the absence of proinflammatory signals resulted in de novo TR development (27). Our findings support the distinct possibility that transient up-regulation of
FOXP3 under proinflammatory conditions may not promote immune-suppressive function in contrast that mediated by preexisting Th3 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 Th3 function. Although such conditions may exist for Th3 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-NTAagarose (Qiagen, Venlo, The Netherlands). Polyclonal antibodies were produced by immunizing rabbits (R&B Rabbittown, WA) every 21 days with 250 μg of His-Foxp3. Hybridoma 2G3 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 antiserum or hybridoma supernatant with protein A or protein G Sepharose affinity chromatography (Amersham Pharmacia Biotech, Cat. No. 17-5766-01). Anti-Foxp3 antibodies were isolated from rabbit antiserum or hybridoma supernatant with protein A or protein G Sepharose affinity chromatography (Amersham Pharmacia Biotech). Anti-Foxp3 antibodies were isolated from rabbit antiserum or hybridoma supernatant with protein A or protein G Sepharose affinity chromatography (Amersham Pharmacia Biotech). Anti-Foxp3 antibodies were isolated from rabbit antiserum or hybridoma supernatant with protein A or protein G Sepharose affinity chromatography (Amersham Pharmacia Biotech).

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 × 10⁶ cells per well (24-well plates) with titrated anti-CD3 (2C11.145 or OKT3) in mouse cell medium (DMEM/10% FBS/0.2% mouse L-glutamine/1 mM sodium pyruvate/penicillin-streptomycin) or human cell medium (RPMI medium 1640/10% human serum/2 mM mouse L-glutamine/10 mM Hepes/1 mM sodium bicarbonate/penicillin-streptomycin).

Flow Cytometry. For staining with Foxp3-specific rabbit polyclonal IgG, cells were fixed in Cytofix/Cytoperm (BD Biosciences) and analyzed on a FACSCalibur or FACS Canto flow cytometer (BD Biosciences). For staining with digoxigenin-labeled Foxp3-specific mouse mAb (3G3-dig), mouse cells were incubated with 10 μg/ml digoxigenin-labeled antibody followed by 5% normal rabbit serum (Jackson ImmunoResearch) (mouse cells) or 5% normal mouse serum (human cells) and then stained with Alexa Fluor 647 or 488-conjugated secondary or biotinylated secondary antibodies. All samples were stained with CD4-peridinin chlorophyll protein (SK3), CD8-PE or CD8-PECy7 (M-38024; BD Biosciences) and other fluorophore-conjugated antibodies (to T.R.T.), the Immunodeficiency Foundation (H.D.O.), a Pfizer Postdoctoral Fellowship in Rheumatology/Immunology (to T.R.T.), the Immunodeficiency Foundation, and the Jeffrey Modell Foundation (H.D.O.). A.Y.R. is a Howard Hughes Medical Institute Investigator.