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Research Article **Immunology**

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Defective regulatory and effector T cell functions in patients with *FOXP3* mutations

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Introduction

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is the approved designation for an autoimmune syndrome previously identified as XPID or XLAAD (1–4). Usually, the onset of the disease is in early infancy, and the course is rapidly fatal, since immunosuppressive therapy has only limited efficacy. The enteropathy manifests with a severe refractory and life-threatening diarrhea, associated with villous atrophy and lymphocytic infiltration of the intestinal mucosa. Other autoimmune manifestations include polyendocrinopathies such as type 1 diabetes (insulin-dependent diabetes mellitus [IDDM]), hypothyroidism, hemolytic anemia, and thrombocytopenia, usually with the presence of autoantibodies. In addition, eczema, typically correlated with elevated titers of IgE in the serum, has also been reported. The disease is rare, but retrospective data on clinical cases of early autoimmune enteritis associated with IDDM, or of neonatal diabetes of unknown origin, suggest that the actual frequency of the disease may be underestimated (5).

IPEX is due to mutations in the *forkhead box protein P3* (*FOXP3*) gene located on chromosome Xp11.23. The gene encodes a protein of 431 amino acids and is a member of the forkhead (FKH) family of transcription factors. In addition to the FKH DNA-binding domain, FOXP3 also contains a leucine zipper and Zn finger binding domain. To date, about 20 different mutations in *FOXP3* have been described in patients suffering from IPEX, with the majority involving the FKH domain, which is essential for DNA binding (1, 6–8). The precise function of FOXP3 has yet to be defined, but studies in the mouse model of the disease, the *Scurfy* mouse, provide evidence that FOXP3 plays a major role in the differentiation of $CD4^+CD25^{high}$ Tregs, in which this gene is constitutively expressed at very high levels (9, 10). Indeed, *Scurfy* mice develop a lethal lymphoproliferative disorder with multiorgan infiltration and autoaggressive phenomena similar to those observed in the absence of Tregs (11, 12). The lack of Tregs, in these mice, strongly suggest that the autoimmunity is due to an uncontrolled expansion of effector T lymphocytes. The observation that adoptive transfer of normal T cells (13) or of an enriched $CD25^+$ T cell subpopulation (14) may be enough to control the disease manifestations is in agreement with the concept that there is a defect in immunoregulatory mechanisms mediated by Tregs.

FOXP3 gene transfer into mouse naive T cells induces the surface expression of CD25 and results in the acquisition of regulatory functions in vitro and in vivo (9, 10, 15, 16). However, there is also evidence that other factors in addition to FOXP3 are required for the optimal function of Tregs and, conversely, that the function of FOXP3 is not restricted to Tregs (17, 18). Indeed, the phenotype of *Scurfy* mice is far more severe than that of mice selectively lacking Tregs, which also develop autoimmunity but do not die as rapidly (11). Interestingly, in human $CD4^+CD25^-$ T cells, expression of FOXP3 can be induced, in both fresh T cells and T cell lines/clones, upon TCR-mediated activation (refs. 19–22 and L. Passerini, unpublished observations) or in the presence of TGF-β (23, 24). These findings suggest a role for FOXP3 in subsets other than the thymus-derived Tregs and indicate that the microenvironment could influence the expression of FOXP3 during an immune response.

Nonstandard abbreviations used: CTLA4, CTL-associated antigen 4; FKH, forkhead; FOXP3, forkhead box protein P3; GITR, glucocorticoid-induced TNF receptor; hIL-2, human IL-2; IDDM, insulin-dependent diabetes mellitus (type 1 diabetes); IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; ND, normal donor; TPA, phorbol myristate acetate.

Conflict of interest: The authors have declared that no conflict of interest exists.

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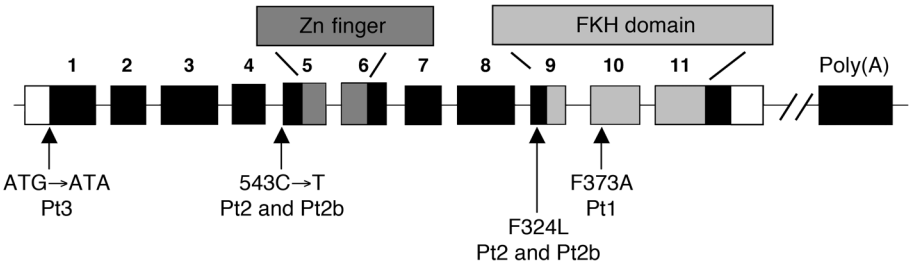

Figure 1
Schematic representation of the genomic organization of the *FOXP3* gene, including the regions encoding each protein domain. Mutations identified in the 4 IPEX patients analyzed are indicated. Pt1, patient 1; Pt2, patient 2; Pt2b, patient 2bis; Pt3, patient 3.

In humans, a correlation among mutations in *FOXP3*, lack of functional Tregs, and IPEX phenotype has not yet been demonstrated. In order to elucidate the underlying immunological defect that causes the autoimmune manifestations in IPEX patients, we studied the phenotype and in vitro function of $CD4^+$ T cells and $CD4^+CD25^{high}$ T cells in 4 children affected by this disease.

Results

Expression of Treg surface markers in T cells from patients with IPEX. We first studied the effect of mutations in *FOXP3* on PBMCs from 4 children (patients 1, 2, 2bis [the brother of patient no. 2], and 3). Mutations and detailed clinical history of the patients are outlined in Methods and in Figure 1. In IPEX patient 1, the absolute lymphocyte number increased during the course of the disease (from 5,500/dl when he suffered from diabetes to 11,000/dl when symptoms of active enteritis appeared), whereas in patients 2, 2bis, and 3, the lymphocyte counts remained stable over time (6,000–8,000/dl). The proportion of T, B, and NK cells and monocytes were within the normal range in patients 1 and 2. When compared with healthy age-matched donors, neither patients had abnormalities in the proportion of naive ($CD45RA^+$) or memory ($CD45RO^+$) T cells, $CD4^+$ or $CD8^+$ T cells, TCRα/β or TCRγ/δ T cells, $CD16^+CD56^+$ NK cells, $CD19^+$ B cells, or $CD14^+$ monocytes (data not shown).

As shown in Figure 2A, the percentage of $CD4^+CD25^+$ cells within the PBMCs was normal in all patients and comparable to that of normal donors (NDs) (25). The MFI of the IL-2 receptor α chain (IL-2Rα) in patients 1 and 2 was comparable to that observed in NDs (range of MFI in NDs, 163–230; $n = 3$), whereas it was lower in patients 2bis and 3. In patients 1 and 2, the proportion of $CD4^+CD25^+$ T cells remained stable at different stages of the disease, and both the percentage of $CD25^{high}$ cells and the MFI were highly reproducible. The MFI of CD4 within the $CD25^+$ population of cells was higher in patients 1 and 3 than in NDs or in the other 2 patients, suggesting the presence of activated T cells. However, in all patients, the Treg-associated markers CTL-associated antigen 4 (CTLA4) and glucocorticoid-induced TNF receptor (GITR) were expressed on $CD4^+CD25^{high}$ T cells at levels comparable overall to those found in age-matched NDs. As expected, these markers were not expressed on the $CD4^+CD25^-$ T cells of ND or IPEX patients. These results indicate that despite mutations in the *FOXP3* gene in IPEX patients, $CD4^+CD25^+$ T cells are present in peripheral blood and they appear phenotypically identical to cells from NDs (26–28).

Further analyses performed in patients 1 and 2 and age-matched NDs revealed that the majority of the $CD4^+CD25^+$ T cells were $CD45RA^+$ and $CD62L^+$ but $HLA\text{-}DR^-$ and $CD69^-$ (Figure 2B), indicating they have a naive resting phenotype, distinct from adult Tregs, which express both CD45RO and HLA-DR (29). To confirm the phenotype of $CD4^+CD25^+$ Tregs in IPEX patients, we FACS sorted $CD4^+CD25^-$ and $CD25^{high}$ T cells from IPEX patients and aged-matched NDs and established T cell lines and T cell clones. As shown in Figure 3, T cell lines derived from $CD4^+CD25^{high}$ T cells isolated from IPEX patients or NDs maintained high expression levels of CD25 in the resting state (12–14 days after restimulation in vitro) and remained positive for CTLA4 and GITR, with the exception of the $CD4^+CD25^+$ T cell line of patient 3, which displayed downregulation of GITR. As expected, all 3 markers were downregulated in resting T cell lines derived from $CD25^-$ cells (Figure 3). Similar results were obtained with T cell clones (data not shown).

Suppressive function of $CD4^+CD25^{high}$ Tregs from IPEX patients. To investigate whether Tregs isolated from IPEX patients had suppressive function, $CD4^+CD25^{high}$ T cells were purified from PBMCs by cell sorting and tested in a suppression assay directly after isolation (patients 2, 2bis, and 3). $CD4^+CD25^-$ T cells from patients and age-matched NDs were sorted in parallel and used as targets of suppression upon activation in the presence of anti-CD3 mAb presented by allogeneic APCs ("weak" TCR stimulus) (Figure 4A) or coated on beads together with anti-CD28 mAb ("strong" TCR stimulus) (Figure 4B). $CD4^+CD25^{high}$ T cells from IPEX patients and NDs were anergic and did not proliferate (Figure 4, A and B) or produce IFN-γ and TNF-α (data not shown) after activation with anti-CD3 mAb in the presence of APCs or anti-CD28 mAb.

Upon activation in the presence of anti-CD3/APCs, $CD4^+CD25^{high}$ T cells freshly isolated from IPEX patients 2 and 2bis suppressed both proliferation (Figure 4A) and IFN-γ and TNF-α production (data not shown) by $CD4^+CD25^-$ T cells from NDs. For patient 2 at a ratio of 1:0.5 (responder/suppressor), the percentage of suppression was slightly lower (44%) compared with that mediated by Tregs from an ND (69%). However, in 3 independent experiments, addition of $CD4^+CD25^{high}$ T cells from patient 2 at this ratio suppressed proliferation by an average of 50% ± 6.5%, compared with 59% ± 19% inhibition by Tregs from an ND. Thus, suppression by ND and patient 2 $CD4^+CD25^{high}$ T cells was comparable overall. $CD4^+CD25^{high}$ T cells from patient 2bis displayed a reduced capacity to suppress proliferation compared with Tregs from an ND tested in parallel, although suppression was still detectable at a 1:0.25 (responder/suppressor) ratio (36% versus 78%) (Figure 4A). Importantly, upon activation in the presence of anti-CD3/anti-CD28–coated beads, a stronger stimulus than anti-CD3/APCs, $CD4^+CD25^{high}$ T cells from both patients 2 and 2bis were clearly impaired in their ability to suppress responder T cells from an ND tested in parallel (Figure 4B).

In contrast to these data obtained using ND responder T cells as targets for suppression, proliferation (Figure 4, A and B) or TNF-α production (data not shown) of autologous $CD4^+CD25^-$ T cells were not suppressed by $CD4^+CD25^{high}$ Tregs from patient 2 and 2bis, independent of the activation conditions used. IL-2 and IFN-γ production by the autologous $CD4^+CD25^-$ T cells used as responders were below detectable levels (see values reported in

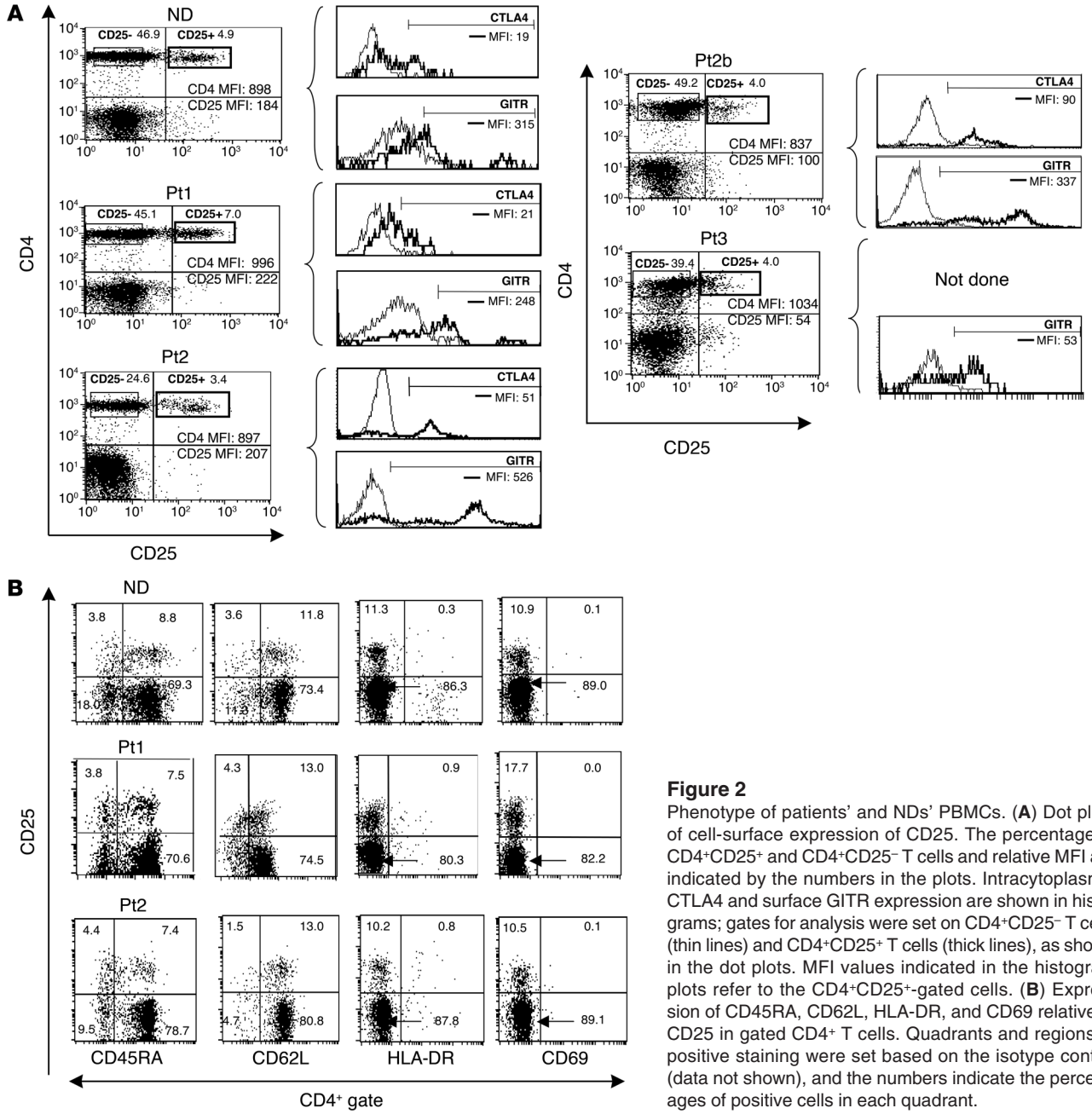

Figure 2
Phenotype of patients' and NDs' PBMCs. (**A**) Dot plots of cell-surface expression of CD25. The percentage of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells and relative MFI are indicated by the numbers in the plots. Intracytoplasmic CTLA4 and surface GITR expression are shown in histograms; gates for analysis were set on $CD4^+CD25^-$ T cells (thin lines) and $CD4^+CD25^+$ T cells (thick lines), as shown in the dot plots. MFI values indicated in the histogram plots refer to the $CD4^+CD25^+$-gated cells. (**B**) Expression of CD45RA, CD62L, HLA-DR, and CD69 relative to CD25 in gated $CD4^+$ T cells. Quadrants and regions of positive staining were set based on the isotype control (data not shown), and the numbers indicate the percentages of positive cells in each quadrant.

Cytokine production in IPEX patients) and therefore could not be used as readout for suppression.

The $CD4^+CD25^{high}$ T cells freshly isolated from patient 3, who has a more severe *FOXP3* mutation, did not display any suppressive activity, toward either ND or autologous responder T cells, even when tested in the presence of a weak (anti-CD3/APC) TCR stimulation (Figure 4A). Due to the limited cell number, only 1 activation condition could be tested in this patient.

These results show that the degree of impairment of Treg-suppressive activity in IPEX patients depends on the type of *FOXP3* mutations and can vary depending on the strength of TCR activation. However, the uniform inability of IPEX $CD4^+CD25^{high}$ T cells to suppress autologous responder T cells points to the potential cellular basis for the autoimmune manifestations in these patients.

Suppressive function of $CD4^+CD25^+$ Treg lines from IPEX patients. Due to the difficulties associated with obtaining blood samples from IPEX patients, we proceeded to in vitro expansion of $CD4^+CD25^{high}$ T cells using a method that preserves suppressive activity, as we previously described (25). We tested $CD4^+CD25^{high}$ T cell lines from patient 1, as fresh cells could not be tested, and $CD4^+CD25^{high}$ T cell lines from patients 2 and 2bis, whose fresh $CD4^+CD25^{high}$ T cells displayed only a partial impairment in their suppressive function. All $CD4^+CD25^{high}$ T cell lines were found to be anergic upon activation with anti-CD3 mAbs (Figure 5). Treg lines obtained from IPEX patients 1, 2, and 2bis suppressed the

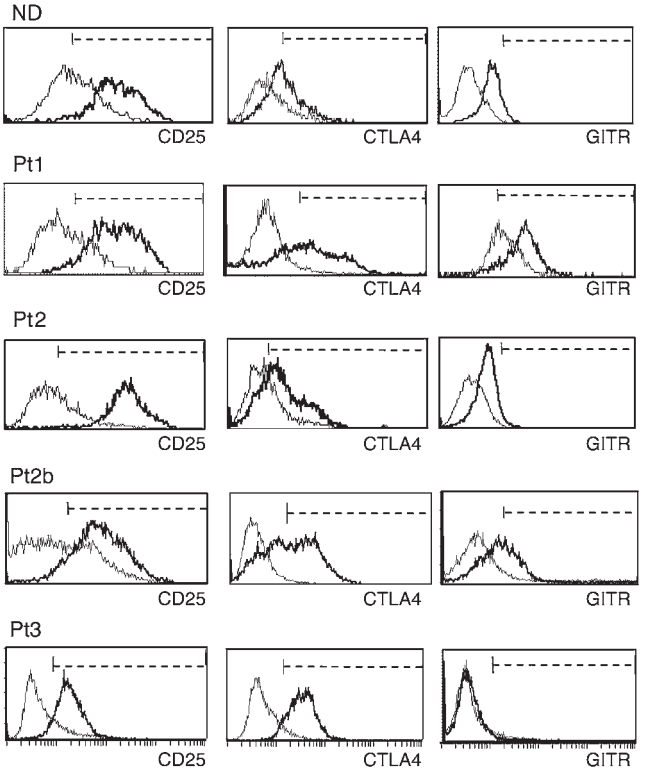

Figure 3
Phenotype of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell lines. Surface expression of CD25 and GITR and intracytoplasmic expression of CTLA4 were determined on T cell lines derived from $CD4^+CD25^-$ (thin lines) and $CD4^+CD25^+$ (thick lines) T cells, isolated by FACS sorting and expanded in vitro. Staining was performed on resting cells (at least 12 days after activation). Regions of positive staining were set based on the isotype control (data not shown).

proliferation of both autologous and ND-derived $CD4^+CD25^-$ T cell lines, even upon activation with plate-bound anti-CD3 mAb, which is known to give a strong TCR signal that can prevent suppression (29, 30). No inhibition of proliferation was detected when $CD4^+CD25^-$ T cell lines were added as controls (Figure 5).

These results show that after in vitro expansion, $CD4^+CD25^{high}$ T cells from IPEX patients do not display an intrinsic defect in suppressive function.

Expression of FOXP3 in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells isolated from IPEX patients. It is well established that *FOXP3* mRNA and protein are highly expressed in $CD4^+CD25^{high}$ Tregs, but in humans, FOXP3 can also be induced in activated $CD4^+CD25^-$ T cells (7, 19–22) and in T cell lines/clones (ref. 31 and our unpublished observations). *FOXP3* mRNA was readily detected in PBMCs, $CD4^+CD25^+$, and $CD4^+CD25^-$ T cell lines from IPEX patients 1 and 2 and NDs (data not shown).

We next evaluated the expression of FOXP3 protein in IPEX patients using T cell lines derived from sorted $CD4^+CD25^{high}$ or $CD4^+CD25^-$ T cells. Figure 6A shows that full-length FOXP3 protein is expressed in $CD4^+CD25^{high}$ T cells from IPEX patients 1 and 2. Note that in human cells, FOXP3 is detected as a doublet and that the upper band corresponds to the canonical sequence, whereas the lower band lacks exon 2 (19). As discussed above, expression of FOXP3 is also induced upon activation of human $CD4^+CD25^-$ T cells. Therefore, nonregulatory $CD4^+CD25^-$ T cell lines from IPEX patients 1 and 2 and NDs also express FOXP3 11–12 days after the last stimulation, although protein levels appear to be higher in $CD4^+CD25^+$ T cell lines. Patient 2bis was not included in this analysis, since he carries the same mutation as patient 2, and therefore FOXP3 protein expression was expected to be identical to that of his brother. In patient 3 we tested expression of FOXP3 protein in activated $CD4^+CD25^-$ T cell lines (Figure 6B), since the $CD4^+CD25^+$ T cell line did not expand to sufficient numbers for protein analysis. In contrast to those of the other patients or NDs, FOXP3 was not detectable in unstimulated or 24- to 48-hour-activated T cell lines. These results demonstrate that the mutations carried by IPEX patients 1, 2, and 2bis do not impair transcription or translation of *FOXP3*, whereas the mutation carried by patient 3 leads to a lack of detectable FOXP3 protein.

Functional DNA-binding capacity of the mutant forms of FOXP3. Although little is known about the targets of FOXP3, it has been reported that this transcription factor represses the activity of the IL-2 promoter (32). We investigated whether the 2 mutant forms of FOXP3 expressed by patients 1 and 2 were impaired in this capacity. Since the mutation in patient 3 resulted in no protein expression, this cDNA was not included in these assays. We established a reporter gene assay with the human IL-2 (hIL-2) promoter and confirmed that coexpression of WT FOXP3 suppressed its activity (Figure 7). Although the fold suppression was on average only 23%, this effect was highly reproducible and statistically significant ($P < 0.0004$). Previous reports used an artificial reporter gene that contained multiple nuclear factor of activated T cell (NFAT) sites (32), which may explain the greater fold suppression. In parallel, we tested the capacity of the mutant forms of FOXP3 of patients 1 and 2 to suppress the IL-2 promoter activity. FOXP3 from patient 1, containing a 2-bp mutation in the DNA-binding FKH domain, was completely unable to suppress the IL-2 promoter. In contrast, FOXP3 from patient 2, which contains mutations outside the DNA-binding FKH domain, was equivalent to the WT protein in its capacity to suppress this promoter. These experiments reveal an important difference in the molecular functionality of FOXP3 between the 2 patients and are consistent with their distinct clinical phenotypes.

Cytokine production in IPEX patients. Since $CD4^+CD25^{high}$ T cells from the patients displayed defective suppression toward autologous $CD4^+CD25^-$ T cells but partially suppressed $CD4^+CD25^-$ T cells from NDs, we next investigated whether effector T cells from IPEX patients were impaired in their proliferation and cytokine production. Proliferative responses of PBMCs from 4 IPEX patients after TCR activation were in the normal range (data not shown). In contrast, TCR-mediated activation of PBMCs (Figure 8A) demonstrated that cells from all 4 IPEX patients had a defect in cytokine production and specifically in their capacity to produce IL-2 and IFN-γ, which were at levels significantly below the minimum values of NDs. Results obtained from IPEX patients were compared with those obtained in 15 NDs of ages ranging from 2 months to 5 years. The defects in IL-2 and IFN-γ production were confirmed in purified $CD4^+CD25^-$ T cells stimulated with anti-CD3/anti-CD28 mAb–coated beads (IL-2 values: patients 1, 2, and 2bis, ≤20 pg/ml; NDs, $n = 5$, 110 ± 31 pg/ml; IFN-γ values: <20 pg/ml in all 3 patients; NDs, $n = 5$, 154 ± 80 pg/ml). Production of TNF-α was also reduced in all patients except patient 3 (P values are reported in Figure 8A). In IPEX patients, levels of IL-5, IL-4, and IL-10 were below the mean values detected in NDs, but the reduction was not statistically significant. The defective cytokine production upon TCR-mediated

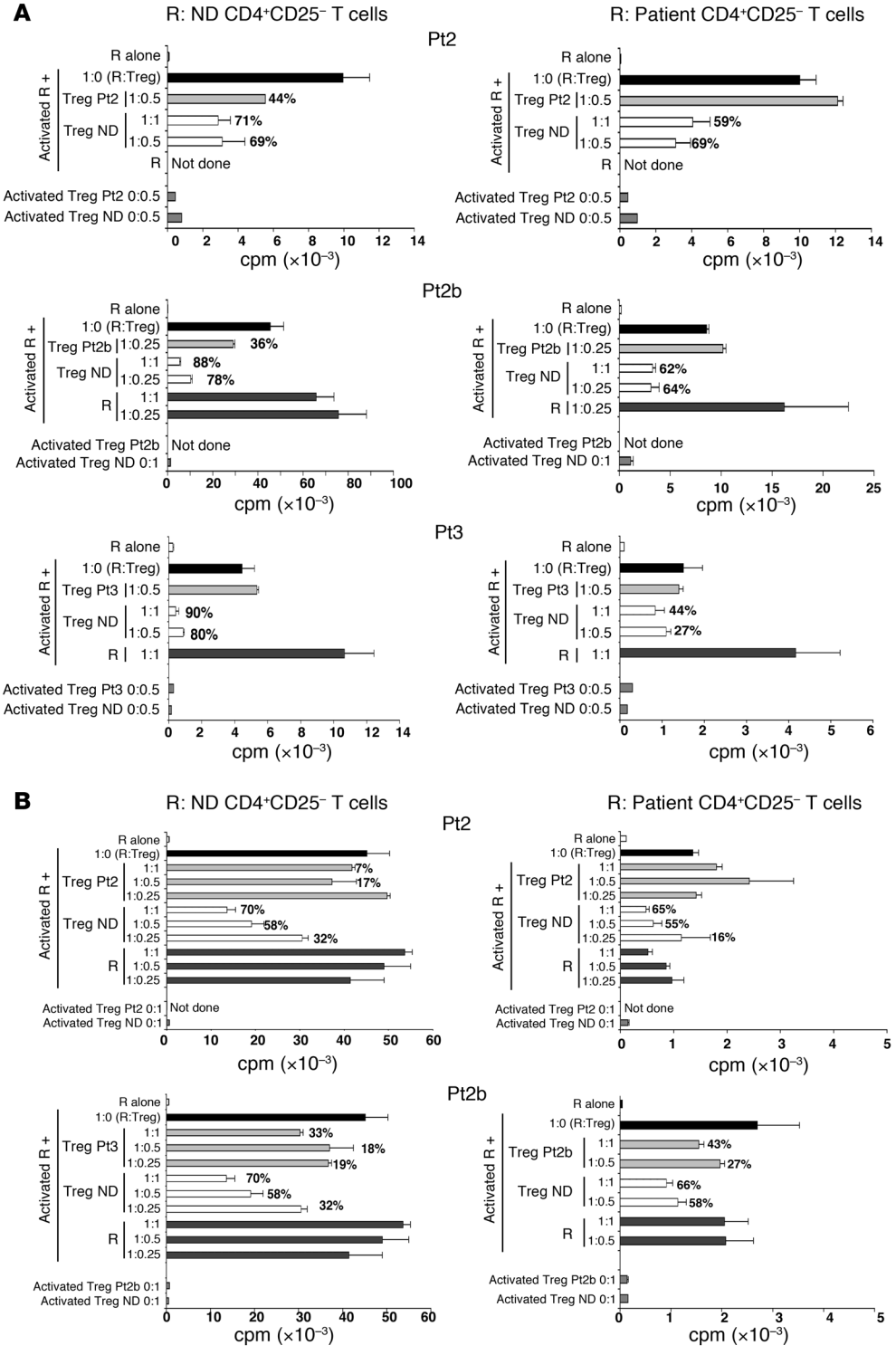

Figure 4
Suppressive activity of patients' CD4+CD25high T cells. The ability of freshly isolated CD4+CD25high T cells of patient 2, patient 2bis, patient 3, and NDs to suppress either ND CD4+CD25− effector T cells or autologous CD4+CD25− effector T cells was assessed (in the case of NDs, effector and suppressor cells were derived from the same individuals). Responder cells were activated either in the presence of 50,000 irradiated (60 Gy) APCs plus soluble anti-CD3 mAb (1 μg/ml) (**A**) or with anti-CD3 plus anti-CD28 mAb–coated beads (**B**) (see Methods for details). CD4+CD25high T cells (Treg) were added to activated responder cells (R), and [3H]thymidine incorporation was measured after 72 hours. Percentages indicate inhibition of proliferation.

activation was partially corrected upon stimulation with phorbol myristate acetate (TPA) and ionomycin, stimuli that bypass the TCR (Figure 8B). Thus, although in IPEX patients the overall cytokine production remained lower compared with that in NDs, stimulation with TPA and ionomycin resulted in production of IL-2, IFN-γ, and TNF-α within normal range. IL-5 secretion of patient 2 was significantly more abundant compared with that of NDs, whereas it was within the normal range in the other patients. Production of IL-4 and IL-10 after TCR-independent activation were within the normal range in all 4 patients. The defect in IL-2 and IFN-γ secretion was confirmed at the mRNA level by quantitative PCR analysis in patients 1 and 2 (data not shown).

Taken together, these data indicate that IPEX patients with different mutations have a common defect in cytokine produc-

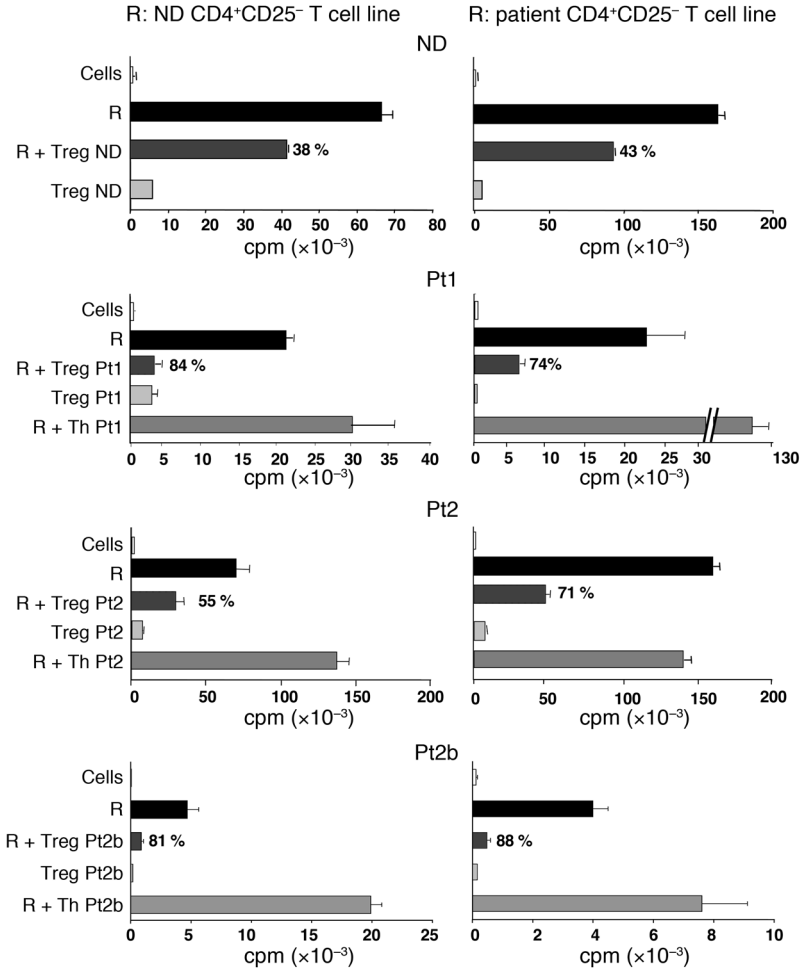

Figure 5
Suppressive activity of patients' CD4+CD25high T cell lines. The ability of CD4+CD25high T cell lines derived from patient 1 (Treg Pt1), patient 2 (Treg Pt2), patient 2bis (Treg Pt2b), or an ND (Treg ND) to suppress CD4+CD25− responders from either allogeneic ND-derived T cell line (left panels) or autologous T cell line (right panels) was assessed. Percentages indicate inhibition of proliferation. Data shown are representative of 3 independent experiments.

tion by PBMCs. This finding suggests that FOXP3 could play an important role in regulating effector T cell functions that are strictly dependent on TCR signaling. Whether the lack of IL-2 and/or IFN-γ production by IPEX effector T cells may result in their inability to appropriately activate the suppressive function of CD4+CD25high T cells, thus contributing to the impaired function of Tregs, remains to be determined.

Discussion

In the murine model of IPEX, the lack of functional Tregs is considered to be the driving force behind the aggressive autoimmunity (7, 9, 10, 15). In the present study, we investigated the effects of different mutations in the *FOXP3* gene on the number, phenotype, and function of Tregs in 4 children affected by IPEX but with diverse clinical phenotypes. Patient 1 presented with a very severe phenotype but preserved FOXP3 protein expression. Patients 2 and 2bis did not have a significant disease and expressed FOXP3 protein. Patient 3 had very severe clinical manifestations and undetectable FOXP3 protein. Peripheral blood CD4+CD25+ T cells were present, expressed the surface markers distinctive of Tregs, and were anergic in all 4 patients. These data indicate that humans with mutations in *FOXP3* do not simply lack Tregs.

Functional analyses of T cell subsets from IPEX patients revealed that CD4+CD25high T cells may either be normally suppressive or impaired to different degrees depending on: (a) the genotype of the target cells; (b) the type of *FOXP3* mutation; and (c) the strength of TCR activation. In the presence of a weak TCR stimulus, which favors detectable suppression in vitro (30), freshly isolated CD4+CD25high T cells from IPEX patients 2 and 2bis suppressed proliferation and cytokine production by ND effector T cells but failed to suppress responses by autologous effector T cells. In the presence of strong TCR activation, however, freshly isolated CD4+CD25high T cells from IPEX patients 2 and 2bis displayed a reduced suppressive capacity compared with ND Tregs, independently of the effector T cells used. In contrast, CD4+CD25high T cells freshly isolated from patient 3 failed to suppress allogeneic or autologous T cells. Interestingly, following in vitro expansion in the presence of feeder cells

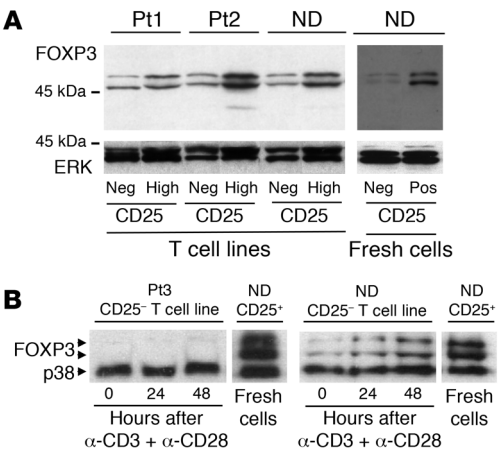

Figure 6
Expression of FOXP3 protein. (**A**) Results of Western blot analysis of FOXP3 protein expression in fresh CD4+CD25− T cells and CD4+CD25high Tregs isolated from a normal control subject and in CD4+CD25− or CD4+CD25high T cell lines from patient 1, patient 2, and an ND are shown. The amount of loaded protein was equivalent in each condition as shown by the anti-ERK staining. (**B**) Results of Western blot analysis of FOXP3 protein expression in T cell lines derived from CD4+CD25− T cells of patient 3 and of an ND, analyzed resting and 24–48 hours after activation, are shown. As a control, results for CD4+CD25+T cells from an ND tested in parallel are also shown. The amount of loaded protein was equivalent in each condition as shown by the anti-p38 staining. Neg, negative; Pos, positive.

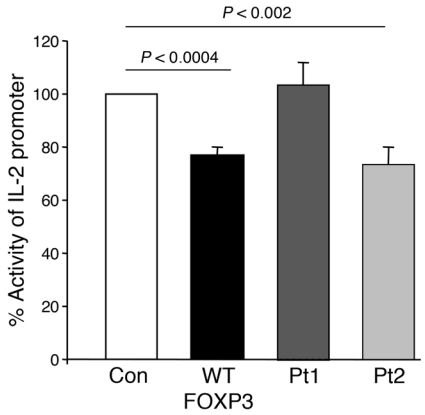

Figure 7
Functional activity of WT FOXP3 and mutants on the IL-2 promoter. Jurkat cells were transfected with a reporter gene construct containing luciferase under the control of the hIL-2 promoter in the presence of an empty vector control (Con), WT *FOXP3*, or mutant *FOXP3* of patients 1 and 2. In all cases, cells were also cotransfected with β-gal under control of the elongation factor 1α promoter. After 16 hours, cells were stimulated with TPA plus ionomycin for an additional 6 hours, then lysed and analyzed for levels of luciferase and β-gal activity. Shown is the percent activity of the IL-2 promoter after normalizing to levels of β-gal, with the empty vector condition set at 100%. Data represent the mean of 4 independent experiments.

and exogenous IL-2, $CD4^+CD25^{high}$ T cell lines from IPEX patients 1, 2, and 2bis displayed normal suppressive functions, irrespective of the source of effector T cells or the strength of activation. Together, our findings suggest that, in contrast to that in the mouse, autoimmunity in human IPEX is not necessarily due to the absence of Tregs but rather to their impaired suppressive function.

A similar defect in the suppressive function of $CD4^+CD25^+$ T cells has also been reported in patients with autoimmune polyglandular syndrome–type II (APS-II), IDDM, and multiple sclerosis (29, 33, 34). In all cases, the mechanistic basis for this defect remains to be clarified. Our results obtained with Tregs from IPEX patients are in line with the notion that the suppressive function of $CD4^+CD25^+$ Tregs varies with the strength of T cell activation. In addition, the observation that the IPEX Treg defect is more profound in the presence of autologous effector T cells points to a possible explanation for their lack of self tolerance. Notably, Tregs from patients with multiple sclerosis (29) have impaired suppressive function at a lower threshold of TCR activation compared with NDs' Tregs and in the presence of autologous T cells (29).

Our data also indicate that the degree of functional defect in IPEX $CD4^+CD25^{high}$ T cells is directly related to the severity of the *FOXP3* mutation, in particular to the levels of FOXP3 expression in T cells, which can vary among patients. The expression of a mutant FOXP3 protein can nevertheless be associated with a severe clinical phenotype (as in patient 1). This could be due not only to the presence of a dysfunctional protein in Tregs but also to a defect in other cell subsets/factors that contribute to the maintenance of self tolerance (15, 17, 19, 35).

Interestingly, in all 4 IPEX patients, a common defect in cytokine production was detected. We found that PBMCs from IPEX patients were significantly deficient in their ability to produce IL-2 and IFN-γ after TCR-mediated activation. These findings suggest a possible involvement of FOXP3 in differential responsiveness to TCR signaling also in effector T cells from IPEX patients. Although $CD4^+CD25^-$ effector T cells from IPEX patients are suppressed normally by Tregs from NDs, their

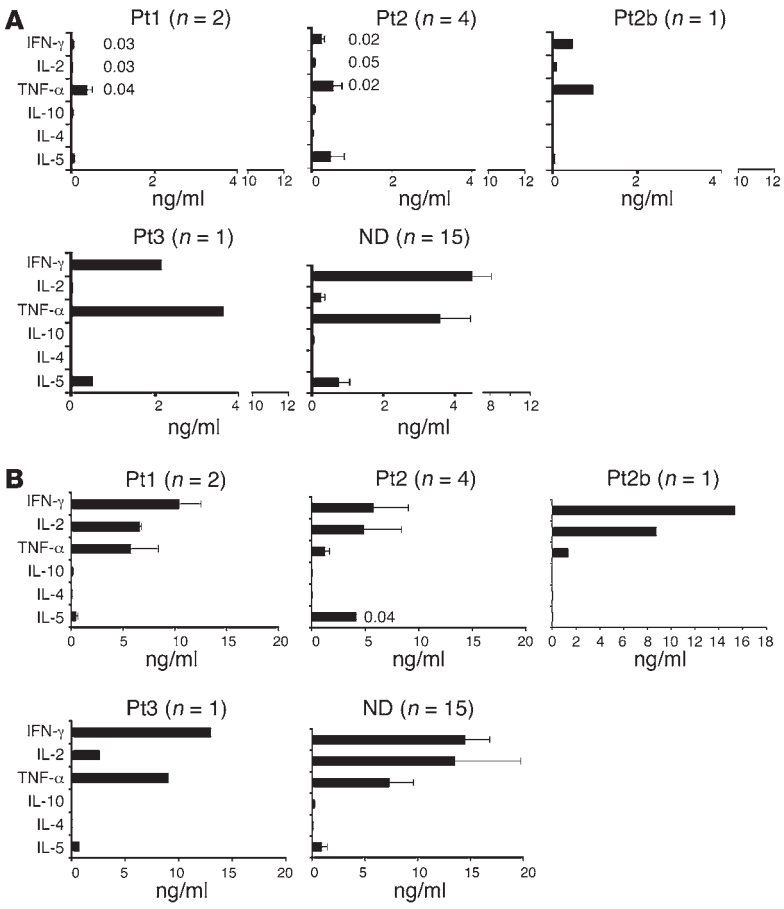

Figure 8
Cytokine production by PBMCs isolated from IPEX patients: determination in cell culture supernatants. PBMCs were stimulated with immobilized anti-CD3 mAb (10 μg/ml) and soluble anti-CD28 mAb (1 μg/ml) (**A**) or with TPA and ionomycin (**B**). Supernatants were collected after 12 (IL-2) or 48 hours (all other cytokines), and cytokine concentrations were determined by cytometric bead array (see Methods for details). The values obtained in patient 1 (n = 2 independent determinations) and patient 2 (n = 4 independent determinations) were compared with those obtained in a cohort of age-matched NDs (n = 15) analyzed in parallel for statistical analysis. One single determination was performed in patients 2bis and 3.

impaired capacity to produce cytokines, and in particular IL-2, may contribute to the lack of suppression by autologous Tregs in vitro and in vivo. It is surprising that all 4 IPEX patients show this profound cytokine defect, despite their disparate clinical phenotypes. Notably, the impairment in cytokine secretion is only detected upon TCR-mediated activation and is corrected by stimuli that bypass the TCR.

The role of IL-2 in immune regulation has recently been reevaluated based on growing evidence that IL-2 signaling plays a fundamental role in the development of $CD4^+CD25^+$ Tregs (36–39). Studies in mouse models have shown that IL-2 is important for peripheral maintenance and function of $CD4^+CD25^+$ Tregs (40–42). Recently, Shevach et al. have demonstrated that transient IL-2 production by effector T cells is required to stimulate the suppressive activity of Tregs (43, 44). In addition, Tregs expand in vivo in the presence of IL-2 (41). In IPEX patients, the defect in IL-2 production does not impair the thymic differentiation of Tregs, but it could be responsible for impairment in their peripheral function/expansion. Our finding that upon in vitro expansion with IL-2, IPEX $CD4^+CD25^{high}$ T cell lines can suppress autologous T cells is in line with this hypothesis. However, many other growth factors or costimulatory molecules could functionally modify IPEX $CD4^+CD25^{high}$ T cells, not only during in vitro culture but also in vivo. For example, IFN-γ secretion by Tregs has also been found to be important for their development in vivo (45). Thus, it cannot be excluded that deficiency in cytokines different from IL-2 or other unknown factors in IPEX patients may contribute to their Treg dysfunction. Indeed, the observation that Tregs from IPEX patients are differentially sensitive to different strengths of activation via the TCR underlines the fact that the molecular and cellular basis of the disease may be multifactorial.

Our study highlights significant differences between the pathological basis for the phenotype of the *Scurfy* mouse and human IPEX patients. In *Scurfy* mice, where a deletion in the *FOXP3* gene leads to absence of the full-length protein and complete lack of Tregs, the lymphoproliferative disorder is more profound than in human IPEX (7, 12, 13). Besides the lack of Tregs, *Scurfy* mice display hyperproduction of cytokines and an increase in the number of memory T cells and in expression of activation markers. In addition, overexpression of FoxP3 in transgenic mice leads to a deficiency in T cells and a lack of cytokine production (9), confirming the immunosuppressive effects of this protein. Here, we provide evidence that *FOXP3* mutations in IPEX patients do not necessarily result in the absence of protein expression, since only the patient with a mutation in the initiating codon failed to express FOXP3. It can be speculated that in humans, distinct FOXP3 mutants may act by altering the function of different associated proteins, leading to heterogeneous biological abnormalities. Moreover, the fact that PBMCs from IPEX patients fail to produce normal levels of cytokines further suggests that in humans, the function of FOXP3 might not be as limited to Tregs as originally hypothesized but could also be important outside the Treg subset (20–24, 35).

The role of FOXP3 in cytokine regulation is unclear. Evidence in the mouse that this protein can suppress the activity of the IL-2 and GM-CSF promoters (32), combined with our data that overexpression of FOXP3 in human naive $CD4^+$ T cells strongly decreases cytokine production (19), indicates that FOXP3 is capable of downregulating cytokine production. This may be related to the capacity of FOXP3 to bind directly to the IL-2 promoter and/or to NFAT and NF-κB, through the DNA-binding FKH domain (46). The finding that the mutation in the DNA binding site of patient 1 reduces the capacity of the protein to suppress the activity of the IL-2 promoter in a transient gene reporter assay is in line with these previous reports. In addition, the fact that the DNA-binding domain of the mutant form of FOXP3 in patient 1 resulted in such a strong suppression of cytokine production ex vivo could be due to altered interactions with other proteins. The preserved suppression of the IL-2 promoter observed in the transient gene reporter assay with FOXP3 from patient 2 is likely due to the fact that mutations are outside the DNA-binding region.

In conclusion, the results of the present study indicate that the pathogenesis of IPEX is not based on an exclusive defect in Tregs. Instead, our findings indicate that although $CD4^+CD25^+$ T cells can be present in normal numbers, their capacity to suppress is impaired depending on the type of mutation, the strength of TCR stimuli, and the genotype of the effector T cells. We hypothesize that, together with the impaired cytokine production in the PBMCs of IPEX patients, the activation and function of Tregs in vivo is impaired due to a combination of defective activation-induced responses by both regulatory and effector T cells. If this hypothesis were correct, cellular therapy with $CD4^+CD25^+$ T cells alone would probably not be sufficient to cure the disease. Currently, bone marrow transplantation is the only cure for IPEX (8), suggesting that it is necessary to completely restore the immune system in order to revert the clinical phenotype. Further studies in IPEX patients using gene transfer and RNA interference technologies will be of crucial importance to understand the molecular events underlying the activation-dependent defects and to identify targets for novel therapeutic approaches.

Methods

Patients. Patient 1 had a severe, life-threatening form of IPEX. He was diagnosed with IPEX following onset of neonatal IDDM at 2 weeks of life and detection of high serum levels of anti-insulin IgG Abs. For genetic analysis, the 11 exons, the intron-exon junctions, and the poly(A) site were amplified and sequenced. In this patient, a double substitution TT→GC in exon 10 at position 1305–1306 of the cDNA (GenBank accession number NM 014009) was detected that resulted in an F373A substitution (Figure 1). This represents a newly identified mutation of the *FOXP3* gene. To rule out the possibility that the mutation is a polymorphism, a panel of 100 control males was analyzed by denaturing high-performance liquid chromatography, and no variations were found. Our results together with the data reported by Wildin et al. (4) indicate that the F373A substitution is not a polymorphism but a missense mutation. The same mutation was detected in the mother, who is therefore a healthy carrier, but not in the healthy brother. At 7 months, he developed refractory diarrhea, and the intestinal biopsy showed complete mucosal atrophy, consistent with the molecular diagnosis. In addition, he had eczema with high serum levels of IgE. Immunological studies were performed on PBMCs isolated at different stages of the disease and always in the absence of immunosuppression. At 9 months of age, shortly after the diagnosis, he was successfully treated with an HLA-identical bone marrow transplant (BMT) from his healthy brother. He is now 3 years old and has full donor chimerism, and, although he did not recover from the IDDM, he has no other symptoms of IPEX.

Patient 2 has a mild form of the disease. At 4 months he presented with severe enteritis, associated with dehydration and hemolytic anemia, and eczema with high serum levels of IgE. All symptoms reverted spontaneously without the need for immunosuppressive drugs. Genetic analysis revealed a mutation consisting of a splice site aberration affecting the 5′

end of exon 5, previously described in other IPEX patients (2, 8). In addition, a nonconservative point mutation in exon 9 consisting of a single T→C nucleotide substitution at position 970 and resulting in an F324L substitution not previously reported was detected. Polymorphism analysis was carried out in 100 healthy subjects, and no abnormalities were found (Figure 1). At the time of this writing, patient 2 is two years old and has no symptoms of the disease, with the exception of sporadic eczema and elevated serum IgE (1,094 IU/ml). The same mutations are present in the 3-and-a-half-year-old brother of patient 2 (patient 2bis), who never had any symptom of the disease, and in the healthy carrier mother.

Patient 3 had severe manifestations of the disease: neonatal IDDM, enteritis, recurrent skin infections, hyper-IgE; his serum was strongly positive for antienterocyte antibodies, and he had mucosal atrophy at the biopsy. After failure of different immunosuppressive treatments, at age 12 months he received an HLA-matched BMT from an unrelated donor. Despite low chimerism (only 6–10% donor-derived cells in the total PBMCs), 32 months after the transplant he showed no symptoms of autoimmune disease. A point mutation in the first codon was identified (ATG→ATA) as the genetic cause of the disease (Figure 1), and it was not present in the mother.

Cell purification. Peripheral blood was obtained upon informed consent from NDs and patients in accordance with local ethical committee approval (Protocol TIGET02, Independent Ethics Committee of HSR). PBMCs were prepared over Ficoll-Hypaque gradients. $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T cells were isolated from patients' PBMCs by FACS sorting, with a resulting purity of greater than 95%. For control cells from NDs, $CD4^+$ T cells were purified by negative selection with the $CD4^+$ T Cell Isolation Kit and subsequently separated into $CD25^+$ and $CD25^-$ fractions by positive selection (Miltenyi Biotec) and were 85–90% pure. To obtain highly purified (>90%) $CD4^+CD25^-$ T cells, the $CD25^-$ fraction was passed over an LD depletion column (Miltenyi Biotec).

In vitro expansion of T cell lines. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were isolated as described above. T cells (2.5×10^5 cells/ml) were stimulated with 0.1 μg/ml phytohemagglutinin (Roche Diagnostics Corp.) in the presence of an allogeneic feeder mixture containing 10^6 PBMCs/ml (irradiated 60 Gy), 10^5 JY cells/ml (irradiated 100 Gy), an EBV–lymphoblastoid cell line expressing high levels of HLA and costimulatory molecules (47). All cultures were performed in X-VIVO 15 medium (Cambrex) supplemented with 5% human serum (Cambrex) and 100 U/ml penicillin/streptomycin (Invitrogen Corp.). Three days after activation, either 40 U/ml, for T cell lines derived from $CD4^+CD25^-$ cells, or 80 U/ml, for T cell lines derived from $CD4^+CD25^+$ cells, of recombinant IL-2 (Chiron Corporation) were added. Cells were periodically split as necessary and fresh medium with IL-2 added. T cell lines were restimulated every 14 days. All experiments on expanded cells were performed at least 12 days after activation.

FACS analysis. Anti-CD4, -CD25, –HLA-DR, -CD69, -CD45RA (all from BD Biosciences — Pharmingen), -CD62L (CALTAG Laboratories), and -GITR (R&D Systems) were directly coupled to FITC or PE. Expression of CTLA4 was determined by intracytoplasmic staining with biotinylated anti-CTLA4 followed by streptavidin-coupled PerCP (BD Biosciences — Pharmingen), as described previously (25).

Western blotting. Cells were lysed in lysis buffer (1% SDS, 10 mM HEPES, and 2 mM EDTA pH 7.4), heated at 95°C for 5 minutes, and sonicated. Protein (10 μg per lane) was separated on 10% SDS-PAGE gels and transferred onto nitrocellulose filters. After blocking in TBS with 0.05% Tween-20 plus 5% nonfat dried milk, membranes were probed with polyclonal rabbit anti-human FOXP3 antiserum (20), followed by goat anti-rabbit HRP Ab (Dako). Either anti-ERK (Cell Signaling Technology) or anti-p38 (Santa Cruz Biotechnology Inc.) was used as control for loading.

Suppression assays. Ninety-six-well round-bottom plates (Corning Inc.) were coated overnight at 4°C with 1 μg/ml (or 10 μg/ml when soluble anti-CD28 at 1 μg/ml was included) anti-CD3 mAbs (Orthoclone; Janssen-Cilag) in 0.1 M Tris pH 9.5 and washed twice with culture medium. Alternatively, responder T cells were stimulated in the presence of CD3-depleted PBMCs (irradiated 60 Gy) and soluble anti-CD3 (1 μg/ml). Responder T cells were plated at 50,000 cells/well in a final volume of 200 μl of complete medium. $CD4^+CD25^+$ T cells were added at 1:1 or 1:0.5 (responder/suppressor), as described previously (25). To test proliferative response to TCR-mediated stimulation, an equal number of $CD4^+CD25^+$ T cells was plated alone in the presence of the indicated stimuli. As stronger TCR-mediated stimulus, beads coated with anti-CD3 and anti-CD28 mAbs (Dynal Biotech) were used. This kind of stimulation resulted in 3- to 10-fold higher proliferative responses of ND T cells compared with those obtained with irradiated APCs with or without anti-CD3 mAb, and therefore it was considered as "strong" activation signal. Responder T cells were plated at 10,000 cells/well in a final volume of 200 μl and activated at a responder/beads ratio of 1:0.6. $CD4^+CD25^+$ Tregs were added at different ratios (from 1:1 to 1:0.25). After 72 hours of coculture, 50 μl of culture supernatant was collected from each well to test for IFN-γ and TNF-α production, and cells were pulsed for 16 hours with 1 μCi per well [^{3}H]thymidine (Amersham Biosciences). Cells were harvested and counted in a scintillation counter.

Transient transfection reporter assays. WT human FOXP3 cDNA, or mutant forms from patients 1 and 2, were cloned into the LXSN retroviral vector. Jurkat T cells were cotransfected using Lipofectamine 2000 (Invitrogen Corp.) with an hIL-2 promoter luciferase reporter plasmid (a kind gift of D. Mueller, University of Minnesota Medical School, Minneapolis, Minnesota, USA) and control (LXSN) or FOXP3-encoding plasmids. A plasmid encoding β-gal under the control of an elongation factor 1α promoter was also included to normalize for transfection efficiency. Activity of the hIL-2 reporter plasmid in the presence of FOXP3 expression was assayed as previously described (19).

Cytokine detection. IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ protein levels in cell culture supernatants were detected using the human Th1/Th2 cytokine cytometric bead array (CBA) system (BD Biosciences). Samples were analyzed on the BD FACSCalibur flow cytometer, according to the manufacturer's instructions. For each cytokine, the minimum detectable level was 20 pg/ml. When quantitative PCR was used to detect cytokines, total RNA was extracted from T cells using Eurozol reagent (Euroclone). RNA (1–5 μg) was reverse transcribed with MMLV reverse transcriptase (Invitrogen Corp.) according to the manufacturer's instructions. IL-2, IFN-γ, TNF-α, and hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA were quantitated using Assay-on-Demand real-time PCR kits (Applied Biosystems). Samples were run in duplicate, and relative expression of cytokines was determined by calculating the difference between threshold cycles for the target and control samples (ΔCt method) after normalization to HPRT expression. A pool of cDNA generated from activated PBMCs of 2 NDs was used as a reference.

Statistics. The Mann-Whitney *U* test was used to evaluate the significance of differences in cytokine levels between patients and NDs. $P < 0.05$ was considered significant. Data are presented as mean ± SEM.

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