

Regulatory T-cell deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-Linked-like disorder caused by loss-of-function mutations in *LRBA*

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Background: A number of heritable immune dysregulatory diseases result from defects affecting regulatory T (Treg) cell development, function, or both. They include immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which is caused by mutations in forkhead box P3 (*FOXP3*), and IPEX-like disorders caused by mutations in IL-2 receptor α (*IL2RA*), signal transducer and activator of transcription 5b (*STAT5b*), and signal transducer and activator of transcription 1 (*STAT1*). However, the genetic defects underlying many cases of IPEX-like disorders remain unknown. **Objective:** We sought to identify the genetic abnormalities in patients with idiopathic IPEX-like disorders. **Methods:** We performed whole-exome and targeted gene sequencing and phenotypic and functional analyses of Treg cells. **Results:** A child who presented with an IPEX-like syndrome and severe Treg cell deficiency was found to harbor a nonsense

mutation in the gene encoding LPS-responsive beige-like anchor (LRBA), which was previously implicated as a cause of common variable immunodeficiency with autoimmunity. Analysis of subjects with LRBA deficiency revealed marked Treg cell depletion; profoundly decreased expression of canonical Treg cell markers, including FOXP3, CD25, Helios, and cytotoxic T lymphocyte-associated antigen 4; and impaired Treg cell-mediated suppression. There was skewing in favor of memory T cells and intense autoantibody production, with marked expansion of T follicular helper and contraction of T follicular regulatory cells. Whereas the frequency of recent thymic emigrants and the differentiation of induced Treg cells were normal, LRBA-deficient T cells exhibited increased apoptosis and reduced activities of the metabolic sensors mammalian target of rapamycin complexes 1 and 2.

Conclusion: LRBA deficiency is a novel cause of IPEX-like syndrome and Treg cell deficiency associated with metabolic dysfunction and increased apoptosis of Treg cells. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Autoantibodies, autoimmunity, forkhead box P3, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, LPS-responsive beige-like anchor, mammalian target of rapamycin complex, T follicular helper cells, T follicular regulatory cells, regulatory T cells

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Supported by National Institutes of Health (NIH) grants R01AI085090 (to T.A.C.) and 1R01AI100315 (to R.S.G.), Children's Health Research Centers Career Development Awards 5K12HD052896 and 1K12HD052896-01A1 (to E.J. and J.C., respectively), a Jeffrey Modell Foundation Translational Research Program Grant Award (to J.C.), grants from the Dubai-Harvard Foundation for Medical Research and the Jeffrey Model Foundation (to R.S.G.) and a Scientific and Technological Research Council of Turkey grant 059B191300622 (to S.K.).

Disclosure of potential conflict of interest: E. Janssen has received research support from the Children's Health Research Center Career Development Award (NIH 5K12HD052896). J. Chou has received research support from the Jeffrey Modell Foundation Translational Research Program Grant Award. T. K. Ohsumi is employed by Seres Health and has received stock options as part of his compensation and received travel support from Seres Health; he was not employed by Seres Health at the time of the study. G. Dbaibo has received research support from the Dubai-Harvard Foundation for Medical Research (R01 AI085090). T. A. Chatila has received research support from the National Institutes of Health. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication May 7, 2014; revised October 15, 2014; accepted for publication October 16, 2014.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2014.10.019>

A number of Mendelian disorders of immune dysregulation and autoimmunity result from defects affecting regulatory T (Treg) cell development and function.¹ The best characterized of these is immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome resulting from deleterious mutations affecting forkhead box P3 (*FOXP3*).²⁻⁴ Patients with IPEX syndrome have a plethora of autoimmune manifestations, including enteropathy; endocrinopathies, especially type 1 diabetes and thyroiditis; and cytopenias.^{5,6} FOXP3 directs the differentiation of Treg cells and is essential for their suppressive functions.⁷⁻⁹ It reinforces and stabilizes transcriptional networks characteristic of Treg cells and maintains Treg cell phenotypic markers.^{10,11} In the absence of a functional FOXP3 protein, the expression of these networks and markers is attenuated, disrupted, or both.

In cohorts of subjects with an IPEX-like phenotype, up to half do not exhibit mutations in *FOXP3*.^{5,12} A number of other gene defects that affect Treg cell function give rise to an IPEX-like phenotype.¹ These include loss-of-function mutations in genes encoding components of the IL-2 receptor pathway, including

Abbreviations used

BCL6:	B-cell lymphoma 6
BEACH:	Beige and Chediak-Higashi syndrome
CD40L:	CD40 ligand
CT:	Computed tomography
CTLA-4:	Cytotoxic T lymphocyte-associated antigen 4
CVID:	Common variable immunodeficiency
4E-BP1:	4E binding protein 1
FOXP3:	Forkhead box P3
ICOS:	Inducible costimulator
IPEX:	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
iTreg:	Induced regulatory T
IVIG:	Intravenous immunoglobulin
LRBA:	LPS-responsive beige-like anchor
mTORC:	Mammalian target of rapamycin complex
PD-1:	Programmed death 1
SLE:	Systemic lupus erythematosus
Tconv:	Conventional T
Teff:	Effector T
T _{FH} :	Follicular helper T
T _{FR} :	Follicular regulatory T
Treg:	Regulatory T
WES:	Whole-exome sequencing

IL2RA and *STAT5b*, and gain-of-function mutations in *STAT1*.^{1,13-16} Nevertheless, defects underlying the majority of cases with an IPEX-like phenotype remain uncharacterized.

To identify novel genetic causes of IPEX-like disorders, we performed whole-exome sequencing (WES) of DNA from candidate subjects. A boy with an IPEX-like disorder, profound Treg cell deficiency, and a normal *FOXP3* gene sequence was found to have a homozygous nonsense mutation in the LPS-responsive beige-like anchor (*LRBA*) gene, which was previously implicated as a cause of common variable immunodeficiency (CVID) with autoimmunity.¹⁷⁻¹⁹ Analysis of other subjects with *LRBA* deficiency also revealed Treg cell deficiency, profoundly decreased canonical Treg cell markers, and impaired Treg cell suppressive function. Patients with *LRBA* deficiency manifested increased levels of autoantibodies against autologous antigens in association with a dramatic increase in the frequency of circulating follicular helper T (T_{FH}) cells and decreased numbers of circulating follicular regulatory T (T_{FR}) cells. Our findings implicate *LRBA* deficiency as a cause of IPEX-like syndrome and suggest that Treg cell dysfunction is a major contributor of the autoimmune manifestations seen in affected subjects.

METHODS**Patients**

Affected subjects with *LRBA* deficiency from 3 families were analyzed. The index cases of families A and B are described herein for the first time, and those of family C have been previously reported.¹⁸ A patient with IPEX syndrome caused by an A384T amino acid substitution in *FOXP3* was identified by means of WES (M. Garcia-Lloret and T. A. Chatila, manuscript in preparation). Patients with a diagnosis of systemic lupus erythematosus (SLE) were identified at the Rheumatology Clinic at Boston Children's Hospital. Control subjects were age group matched. All study participants were recruited, with written informed consent approved by local institutional review boards. Studies at Boston Children's Hospital were conducted under approved protocol #04-09-113R.

Antibodies and flow cytometry

Information on the antibodies used is provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Whole blood was incubated with mAbs against surface markers for 30 minutes on ice. Intracellular staining with FOXP3, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), Helios, Bcl6, and CD40 ligand (CD40L) mAbs was performed by using eBioscience Fixation/Permeabilization buffer (eBioscience, San Diego, Calif), according to the manufacturer's instructions. Intracellular staining for pS6, phosphorylated 4E binding protein 1 (4E-BP1), and pAKT (S473) was performed on freshly isolated PBMCs either left untreated or stimulated with anti-CD2/CD3/CD28 mAb-coated beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 4 or 12 hours with eBioscience Fixation/Permeabilization buffer. Annexin V (BioLegend, San Diego, Calif) staining was performed on PBMCs by using BioLegend Annexin V binding buffer, according to the manufacturer's instructions. Data were collected with a Fortessa cytometer (BD Biosciences, San Jose, Calif) and analyzed with FlowJo software (TreeStar, Ashland, Ore).

WES

WES was performed on genomic DNA of the probands of families A and B, their parents, and their healthy siblings through AxSeq (Rockville, Md). The Agilent SureSelect Target enrichment kit was used for exon capture (Agilent Technologies, Santa Clara, Calif). Paired-end sequencing was performed with an Illumina HiSeq2000 instrument (Illumina, San Diego, Calif), which generated 100 base pair reads. Average coverage of the exome was 183-208 times. Analysis of WES data was performed with MolBioLib.²⁰ Variants were identified by using the Genome Analysis Toolkit with standard filtering parameters according to Genome Analysis Toolkit Best Practices Recommendations.²¹ Variant calls with a read coverage of 2 times or less and a Phred-scaled SNP quality of 20 or less were eliminated.

Immunoblotting

Protein lysates derived from fibroblasts (patient P1) or lymphocytes (patients P2, P3, P5, and P6) were resolved on SDS-PAGE gels and transferred to nitrocellulose filters, as previously described.²² Immunoblots were carried out with polyclonal rabbit anti-*LRBA* antibodies (Sigma-Aldrich, St Louis, Mo). The blots were reprobed with mouse anti- β -actin mAb (Clone C4; Millipore, Billerica, Mass).

Treg cell suppression assays

PBMCs were isolated from whole blood by means of centrifugation over Ficoll-Hypaque gradients. CD4⁺ T cells were separated by using negative selection with magnetic beads (Miltenyi Biotec). Treg cells were purified by means of cell sorting for CD4⁺CD25⁺CD127^{low} T cells. The suppressor function of Treg cells of both patients and control subjects was tested against CD4⁺CD25⁺ effector T (Teff) cells from a common third-party donor. Teff cells were isolated by depleting donor PBMCs of CD25⁺ T cells with anti-CD25 beads (Miltenyi Biotec). They were labeled with CellTrace Violet dye (Life Technologies, Carlsbad, Calif) to monitor cell divisions by using flow cytometry and resuspended in complete media (RPMI-1640 with 10% FCS, 10 μ M/L HEPES buffer, 1 mmol/L pyruvate, nonessential amino acids, and penicillin/streptomycin). The cells were placed in round-bottom wells of 96-well tissue-culture plates at 0.5 to 1 $\times$ 10⁴ cells per well alone or together with anti-CD2/CD3/CD28 mAb-coated beads. Treg cells were added at a 1:1 ratio to Teff cells, as indicated. On day 4, Teff cells were evaluated by using flow cytometry for cell divisions, as revealed by means of dye dilution. The percentage of Teff cells with more than 1 division was determined. The percentage of suppression was calculated by dividing the percentage of divided Teff cells from each suppressed culture by the percentage of divided Teff cells in the nonsuppressed cultures and subtracting this fraction from 1.

In vitro generation of induced Treg cells

CD4⁺ T cells were isolated from PBMCs by using negative selection with magnetic beads (Miltenyi Biotec). Naive CD3⁺CD4⁺CD25⁺CD45RO⁺CD45RA⁺

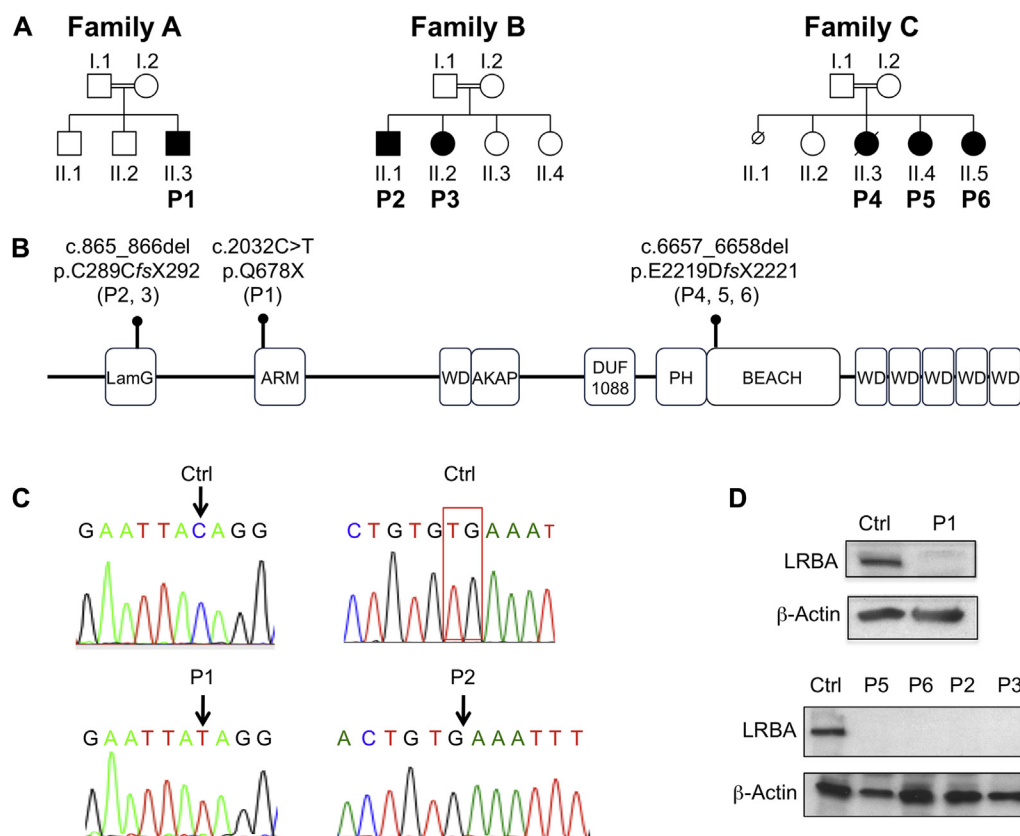


FIG 1. **A**, Pedigrees of the LRBA-deficient families. Generations are designated by Roman numerals (I–II), and subjects are designated by Arabic numerals. Double lines connecting parents indicate consanguinity. Probands in the respective families are indicated as P1 to P6. *Squares*, Male subjects; *circles*, female subjects; *solid symbols*, patients. **B**, Schematic representation of LRBA protein showing the respective domains and locations of patient mutations. **C**, Sanger sequencing fluorograms showing genomic DNA mutations of patient P1 and P2 compared with the equivalent DNA sequences of healthy control subjects (Ctrl). **D**, Western blot analysis of LRBA protein expression in fibroblasts of patient P1 and lymphocytes of patients P2, P3, P5, and P6 compared with those of control subjects. The blots were reprobed for β -actin as a positive control.

cells were purified from CD4⁺ T cells by means of cell sorting on a FACS Aria (purity >98%). Naive CD4 T cells were resuspended in complete medium and seeded at a concentration of 5×10^4 cells per well of a 96-well plate and stimulated with anti-CD2/CD3/CD28 mAb-coated beads (Miltenyi Biotec) and 5 ng/mL recombinant human TGF- β 1 (R&D Systems, Minneapolis, Minn). After 4 days, intracellular staining for FOXP3 was performed.

Cytokine and autoantibody assays

For IL-10 assays, cell-sorted Treg cells were seeded in round-bottom wells at 5×10^3 cells per well and either left untreated or treated with anti-CD2/CD3/CD28 mAb-coated beads or phorbol 12-myristate 13-acetate/ionomycin (20 ng/mL and 1 μ mol/L, respectively). The supernatants were harvested 4 days later and analyzed for IL-10 by using ELISA (eBioscience). For autoantibody detection, plasma aliquots from patients and control subjects were analyzed with microarrays spotted with 84 autoantigens (University of Texas Southwestern Medical Center, Genomic and Microarray Core Facility), as previously described.²³ Data were normalized to healthy control values. Anti-nuclear antigens and double-stranded DNA antibodies were measured by using ELISA (GenWay Biotech, San Diego, Calif, and Alpha Diagnostics, San Antonio, Tex).

Statistical analysis

Aggregate results are presented as means $\pm$ SEMs. Comparison between groups was carried out with the Student unpaired 2-tailed *t* test and 2-way

ANOVA with Bonferroni posttest analysis, as indicated. Differences in mean values were considered significant at a *P* value of less than .05.

RESULTS

In family A (Fig 1 and see Fig E1 in this article's Online Repository at www.jacionline.org) the index case (P1) is a 2-year and 10-month old Saudi Arabian boy who presented at the age of 3 months with protracted diarrhea after his first childhood vaccination. Evaluation was negative for celiac disease, and the pathology was consistent with an autoimmune enteropathy. Within a few weeks, he was given a diagnosis of type 1 diabetes with positive insulin antibodies and hypothyroidism with positive thyroid antibodies. He also had a Coombs-positive autoimmune hemolytic anemia. In addition, he had recurrent episodes of sepsis, including *Pseudomonas aeruginosa* septicemia and *Streptococcus pneumoniae*-related septic shock. Analysis of his immunologic status revealed reduced T-cell proliferative responses to PHA (approximately 50% of normal), normal B-cell numbers, and increased immunoglobulin levels in the setting of low normal specific antibody responses (see Table E1 in this article's Online Repository at www.jacionline.org). Analysis of his *FOXP3* gene sequence did not reveal the presence of deleterious mutations.

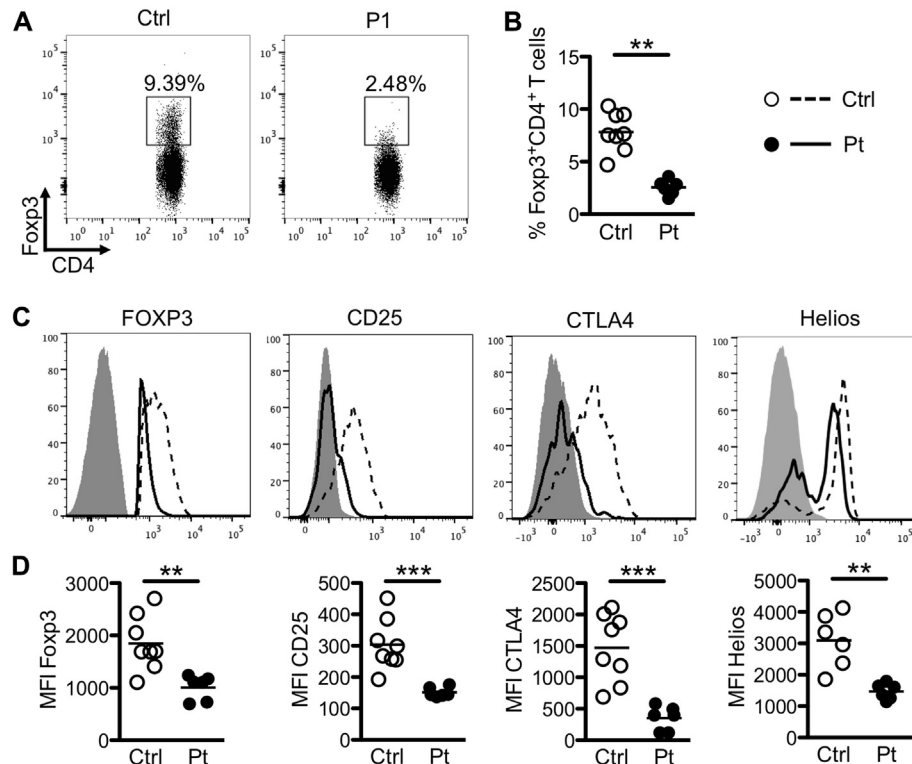


FIG 2. LRBA deficiency leads to a defect in Treg cell frequency and phenotype. **A**, Representative dot plot analysis of FOXP3 expression in patient P1 versus a control subject. **B**, Percentage of CD4⁺FOXP3⁺ T cells in peripheral blood of healthy control subjects ($n = 8$, open circles) and LRBA-deficient patients ($n = 6$, solid circles). **C**, Representative flow cytometric histogram plots of FOXP3, CD25, CTLA4, and Helios expression in FOXP3⁺CD4⁺ T cells from control subjects (dotted line) and LRBA-deficient patients (solid line). The gray area represents control isotype staining. **D**, Mean fluorescence intensity (MFI) of the respective Treg cell marker in patients and control subjects. ** $P < .01$ and *** $P < .001$, unpaired 2-tailed Student t test.

In view of his consanguineous heritage, we undertook WES to identify gene variants that were homozygous in the patient, heterozygous in his mother, and either heterozygous or absent in his healthy brother. This filtering approach identified 18 candidate variants, which were nonsynonymous, absent from dbSNP, and not present in the homozygous state in any of our 80 in-house Middle Eastern exomes (see Table E2 in this article's Online Repository at www.jacionline.org). Although the majority of these variants were scored as benign by using PolyPhen and/or SIFT protein function prediction algorithms, the one variant that stood out in relation to its deleterious effect on the immune system involved *LRBA*.¹⁷⁻¹⁹ The patient had a homozygous c.2032 C>T substitution in exon 16 of *LRBA*, which encodes the third armadillo domain of LRBA, resulting in the replacement of amino acid glutamine 678 with a stop codon (Q678X) and absent protein expression (Fig 1). The mutation was confirmed by using Sanger sequencing, and both parents were heterozygous carriers of the mutation (see Fig E1).

LRBA deficiency was also found in a second newly described Lebanese family with 2 siblings (Family B; P2 and P3), whose clinical histories were notable for recurrent infections and immune dysregulation (see the Case reports section in this article's Online Repository at www.jacionline.org). A homozygous exonic deletion in *LRBA* (c.865_866del) was the lead candidate variant identified by means of WES under the aforementioned filtering conditions (see Table E3 in this

article's Online Repository at www.jacionline.org). It was confirmed by means of Sanger sequencing and resulted in absent protein expression (Fig 1). Both parents and the patients' 2 clinically unaffected siblings (IL3 and IL4; Fig 1, A) were heterozygous carriers of the mutation (see Fig E2 in this article's Online Repository at www.jacionline.org). We also studied 3 previously described Saudi Arabian siblings (P4, P5, and P6; Family C) with LRBA deficiency caused by a homozygous deletion in the beige and Chediak-Higashi syndrome (BEACH) domain of *LRBA* that abolished protein expression (Fig 1, A, B, and D).¹⁸ The clinical and laboratory findings of these patients are detailed in Table E1.

In view of their immunodysregulatory phenotypes, most notably the IPEX-like disease of patient P1, we examined our cohort of LRBA-deficient patients for evidence of Treg cell abnormalities. Flow cytometric analysis of peripheral blood Treg cells of patient P1 demonstrated a markedly reduced number of CD4⁺FOXP3⁺ Treg cells (Fig 2, A). Analysis of the 5 other patients with LRBA deficiency revealed that they all share a profound decrease in Treg cell frequency in the peripheral blood (control subjects: $7.54\% \pm 0.64\%$ vs patients: $2.45\% \pm 0.29\%$; Fig 2, B). Importantly, expression of several canonical Treg cell markers, including FOXP3, CD25 (IL-2RA), CTLA-4, and Helios, was profoundly decreased in LRBA-deficient patients relative to control subjects (Fig 2, C). Thus LRBA deficiency was associated with decreased numbers and an aberrant phenotype of Treg cells.

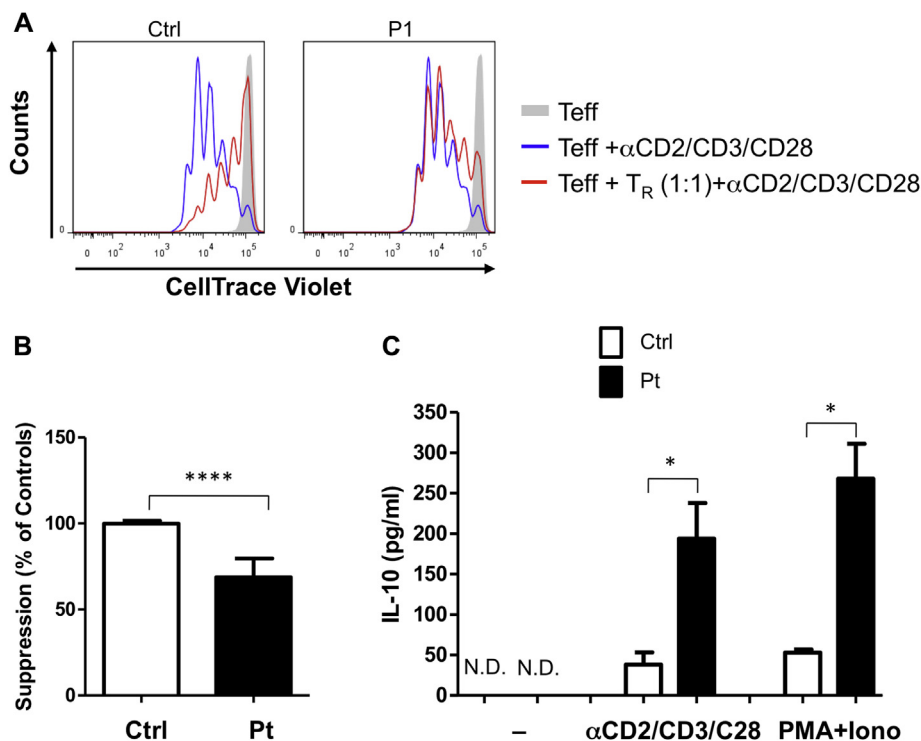


FIG 3. LRBA-deficient Treg cells have decreased *in vitro* suppressive ability. **A**, Flow cytometric analysis of CellTrace Violet proliferation dye dilution in Teff ($CD4^+CD25^-$) cells stimulated with anti-CD2/CD3/CD28 mAb-coated bead in the absence or presence of Treg (T_R) cells ($CD4^+CD25^+CD127^{low}$) from healthy control subjects or LRBA-deficient patients. **B**, Cumulative results of *in vitro* suppression assays in patients and control subjects ($n = 6$ subjects per group). **** $P < .0001$, unpaired 2-tailed Student *t* test. **C**, IL-10 production in Treg cells from patients and control subjects at baseline and after stimulation with anti- α CD2/CD3/CD28 mAbs or phorbol 12-myristate 13-acetate plus ionomycin (PMA + Iono) for 4 days. N.D., Not detected. * $P < .05$, 2-way ANOVA with posttest analysis.

We further analyzed the effect of LRBA deficiency on Treg cell suppressive function using an *in vitro* suppression assay of T-cell proliferation to mitogenic stimulation. Treg cells were isolated by means of cell sorting of $CD4^+CD25^+CD127^{low}$ Treg cells. They were confirmed by using intracellular staining to be greater than 90% positive for FOXP3, which is indicative of their Treg cell lineage (data not shown). Equal numbers of patient and control Treg cells were added to an equal number of control $CD4^+CD25^-$ Teff cells loaded with the proliferation dye CellTrace Violet and treated with a mitogenic combination of anti-CD2/CD3/CD28 mAbs. LRBA-deficient Treg cells manifested decreased suppression of T-cell proliferation, measured by using tracer dye dilution in target Teff cells compared with control Treg cells, which is indicative of their impaired function (Fig 3, A and B). Defective *in vitro* suppression by LRBA-deficient Treg cells could not be ascribed to decreased IL-10 production, the deficiency of which in Treg cells is associated with colitis.²⁴ IL-10 production was increased in Treg cells from patients compared with those from control subjects (Fig 3, C).

Treg cell deficiency is associated with dysregulated T-cell activation and skewing toward a memory phenotype. Consistent with immune dysregulation, LRBA deficiency resulted in skewing of peripheral CD4 and CD8 T cells toward a memory ($CD45RA^-CD45RO^+$) phenotype (control subjects vs LRBA-deficient patients: $59.5\% \pm 2.8\%$ vs $86\% \pm 2.1\%$ for CD4 T cells and $29.4\% \pm 4.2\%$ vs $50.3\% \pm 6.2\%$ for CD8 T cells,

respectively; Fig 4, A-D). The frequency of naive $CD45RA^+CCR7^+CD8^+$ T cells was decreased in LRBA-deficient patients (control subjects vs LRBA-deficient patients: $10.4\% \pm 3\%$ vs $3\% \pm 0.7\%$). However, there was no difference in the frequency of $CD8^+CD45RA^+CCR7^-$ ($CD45RA^+$ effector memory) T cells compared with that seen in control subjects (Fig 4, E and F).

LRBA-deficient patients have diverse autoimmune phenomena, including autoimmune hemolytic anemia, thrombocytopenia, thyroiditis, and end-organ inflammation, including colitis and pneumonitis.¹⁷⁻¹⁹ Using an array of 84 autoantigens, we determined the spectrum of circulating autoantibodies in the 6 LRBA-deficient patients compared with a patient with IPEX with a missense mutation in the forkhead domain of *FOXP3* (p.A384T), an adolescent patient with SLE, and 5 healthy control subjects. When normalized to the results of the control subjects, the LRBA-deficient patients had increased autoantibodies present in their plasma. This was especially true for patient P1, whose phenotype and autoantibody profile were similar to those of the patient with true IPEX syndrome (Fig 5, A, and data not shown). There was a predominance of cytoplasmic- and RNA-directed autoantibodies in LRBA-deficient patients. Similar to patients with IPEX and unlike those with SLE, they were negative for antinuclear and anti-double-stranded DNA antibodies (Fig 5, A; see Fig E3 in this article's Online Repository at www.jacionline.org; and data not shown).

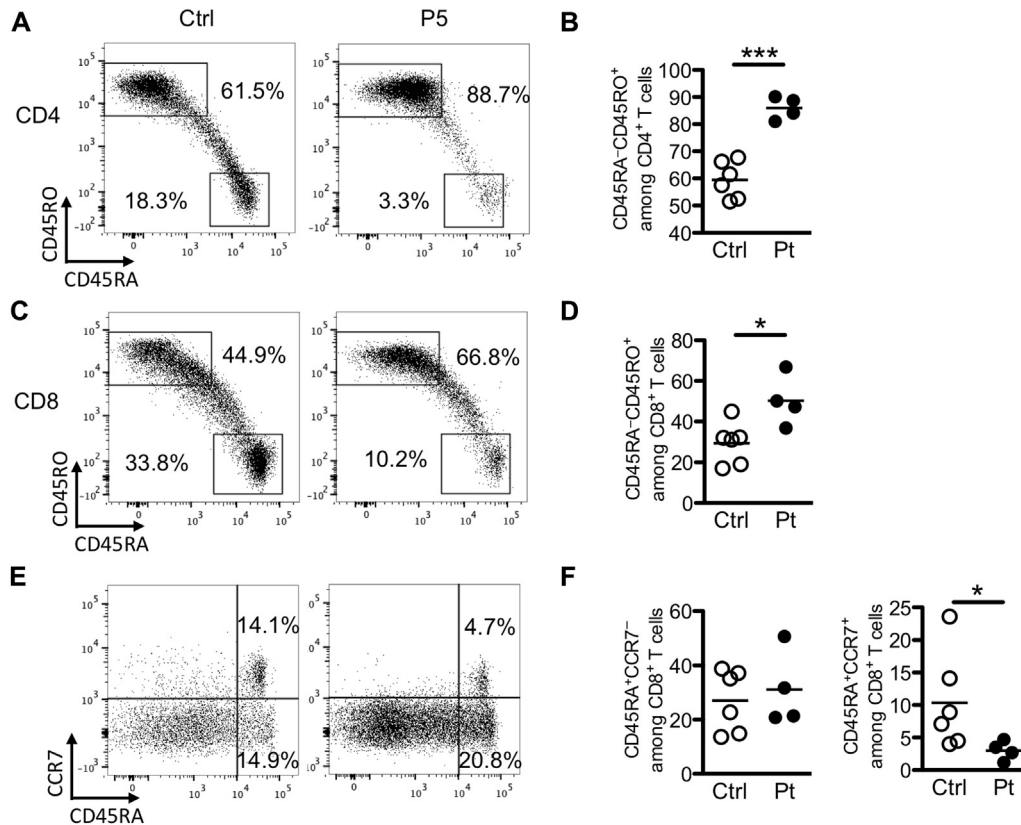


FIG 4. Increased frequency of memory T cells in LRBA-deficient patients. **A**, Flow cytometric analysis of circulating CD45RO⁺CD45RA⁻ and CD45RO⁺CD45RA⁺ CD4⁺ T cells in an LRBA-deficient patient and a control subject. **B**, Frequency of activated CD45RO⁺CD45RA⁻ cells within the peripheral blood CD4⁺ T-cell pool of LRBA-deficient patients and control subjects. **C**, Flow cytometric analysis of circulating CD45RO⁺CD45RA⁻ and CD45RO⁺CD45RA⁺ CD8⁺ T cells in an LRBA-deficient patient and a control subject. **D**, Frequency of activated CD45RO⁺CD45RA⁻ cells within the peripheral blood CD8⁺ T-cell pool of LRBA-deficient patients and control subjects. **E**, Flow cytometric analysis of circulating CD45RA⁺ effector memory (TEMRA) CD45RA⁺CCR7⁻ and naive CD45RA⁺CCR7⁺ CD8⁺ T cells in an LRBA-deficient patient and a control subject. **F**, Frequency of circulating TEMRA CD45RA⁺CCR7⁻ and naive CD45RA⁺CCR7⁺ cells within the peripheral blood CD8⁺ T-cell pool of LRBA-deficient patients and control subjects. * $P < .05$ and *** $P < .001$, Student unpaired 2-tailed t test.

A specialized subset of Treg cells, termed T_{FR} cells, regulates the ability of T_{FH} cells to provide support for cognate B cells in developing germinal centers and mounting T cell-dependent antibody responses.²⁵⁻²⁸ T_{FR} deficiency, as seen in patients with IPEX and mice with FOXP3 deficiency, results in dysregulated expansion of T_{FH} cells and germinal center B cells, leading to intense autoantibody formation.^{25,29} Both T_{FH} and T_{FR} cells express the chemokine receptor CXCR5 and also have high expression of the inhibitory receptor programmed death 1 (PD-1).²⁵⁻²⁸ Circulating T_{FR} (CD4⁺FOXP3⁺CXCR5⁺PD-1^{hi}) and T_{FR} (CD4⁺FOXP3⁺CXCR5⁺PD-1^{hi}) cells are found in the peripheral blood, and the former are detected in greater abundance in patients with diseases associated with intense autoantibody production, such as SLE, myasthenia gravis, and juvenile dermatomyositis.³⁰⁻³² The gating strategy for detecting circulating T_{FR} and T_{FR} cells and the specificity of the staining are shown in Figs E4 and E5 in this article's Online Repository at www.jacionline.org, respectively. There was a striking increase in the frequency of T_{FR} cells in the peripheral blood of LRBA-deficient patients (control subjects: 4.9% $\pm$ 0.6% vs LRBA-deficient patients: 31.2% $\pm$ 5.3%; Fig 5, B and C). The

patient's CXCR5⁺PD-1^{hi} cells expressed high levels of B-cell lymphoma 6 (BCL6) protein, inducible costimulator (ICOS) protein, and CD40L, which is consistent with their T_{FR} phenotype (Fig 5, D). Within the T_{FR} cell population, the frequency of FOXP3⁺T_{FR} cells was significantly decreased in patients relative to that seen in control subjects (control subjects: 10.3% $\pm$ 2% vs patients: 3.5% $\pm$ 0.6%; Fig 5, E and F). These results are consistent with dysregulated T_{FR} and compromised T_{FR} cell compartments in LRBA-deficient subjects.

To determine whether LRBA deficiency impaired induction of FOXP3 expression, we analyzed the capacity of LRBA-sufficient and deficient naive CD4⁺ T cells to differentiate into induced regulatory T (iTreg) cells on stimulation with anti-CD2/CD3/CD28 mAbs in the presence of TGF- β . CD4⁺ T cells from patients and control subjects were enriched by using magnetic separation, and naive CD4⁺ T cells were purified to greater than 98% purity by means of cell sorting (Fig 6, A, and data not shown). LRBA-sufficient and deficient T cells were found to be equivalent in their capacity to differentiate into iTreg cells that equally expressed high levels of FOXP3 (Fig 6, B and C).

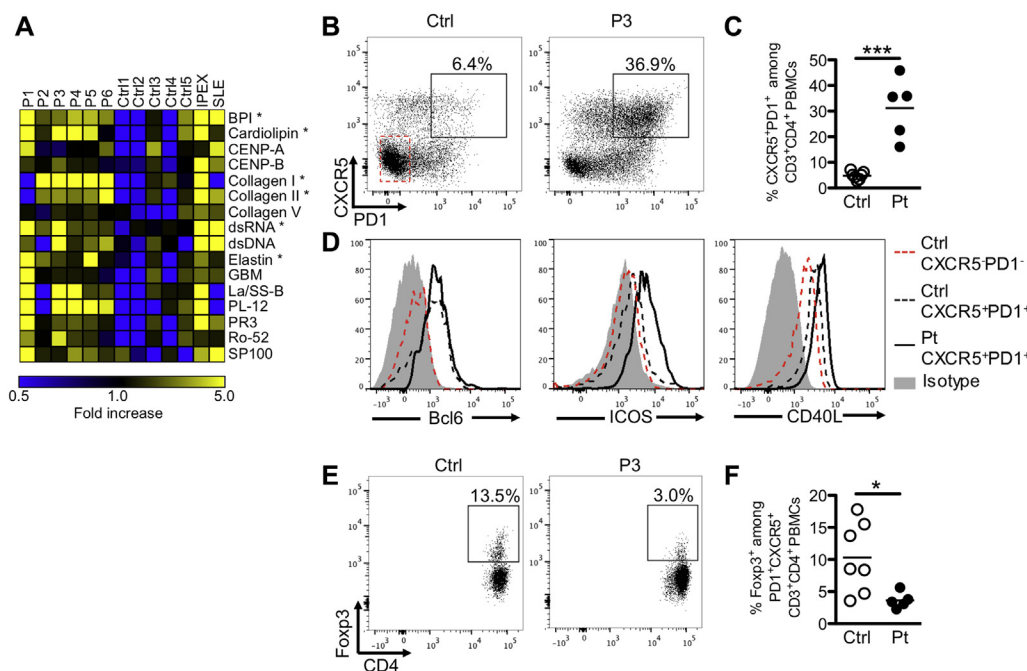


FIG 5. Increased autoantibodies and dysregulated T_H cell response in LRBA-deficient patients. **A**, Heat map display of autoantibody reactivity against self-antigens in sera of LRBA-deficient patients and control subjects, a patient with IPEX, and a patient with SLE. A value of 1 (black) is equal to the control average + 1 SD. **B**, Flow cytometric analysis of circulating T_H ($CD4^+CXCR5^+PD-1^{hi}$) cells in patients and control subjects. **C**, Frequency of T_H cells within the peripheral blood $CD4^+$ T cells of LRBA-deficient patients and control subjects. **D**, Flow cytometric overlays of the T_H markers BCL6, ICOS, and CD40L within the peripheral blood $CD4^+CXCR5^+PD-1^+$ cell pool of LRBA-deficient patients and control subjects. **E**, Flow cytometric analysis of circulating T_H cells. T cells within the $CD4^+CXCR5^+PD-1^{hi}$ gate were analyzed for Foxp3 expression. **F**, Frequencies of T_H cells within the peripheral blood $CD4^+CXCR5^+PD-1^+$ cell pool. * $P < .05$ and *** $P < .001$, Student unpaired 2-tailed t test.

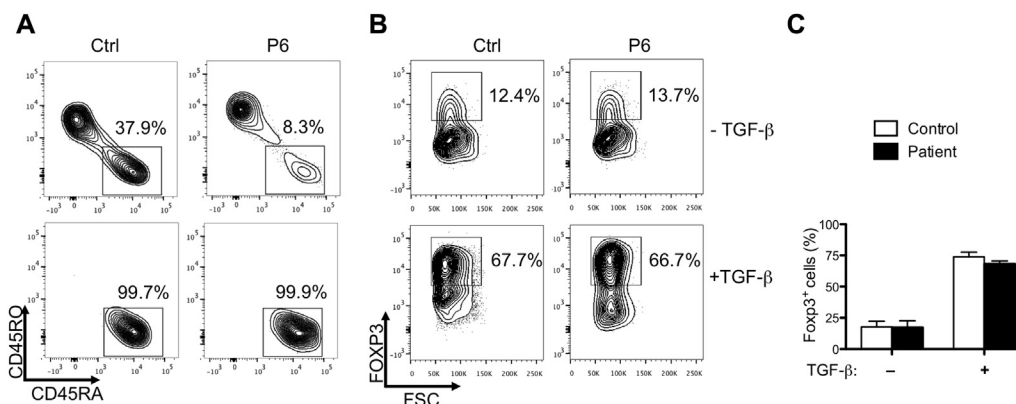


FIG 6. LRBA deficiency does not affect *in vitro* generation of FOXP3 $^+$ iTreg cells. **A**, Representative flow cytometric analyses of CD45RA/CD45RO markers in purified $CD4^+$ T cells (upper panel) and cell-sorted $CD3^+CD4^+CD25^-CD45RO^-CD45RA^+$ T cells (lower panels) of a control subject and an LRBA-deficient patient. **B**, Representative flow cytometric analyses of Foxp3 expression in T cells sorted as in Fig 6, A, and stimulated in culture with anti-CD2/CD3/CD28 mAbs in the absence or presence of TGF- β 1. **C**, Collated frequencies of FOXP3 $^+$ iTreg cells in control subjects and LRBA-deficient patients ($n = 4-5$ per group). Results are representative of 2 independent experiments.

Given the normal differentiation of LRBA-deficient T cells into iTreg cells, we examined the turnover of peripheral blood Treg and $CD4^+$ conventional T (Tconv) cells in patients versus control subjects. Results revealed that both Treg and Tconv cells of LRBA-deficient patients exhibited increased Annexin V staining compared with those of control subjects, which is indicative of

increased apoptosis (control subjects vs LRBA-deficient patients: $6.3\% \pm 0.6\%$ vs $13.7\% \pm 3.2\%$ for $CD4^+$ Tconv cells and $15.7\% \pm 1.3\%$ vs $22.6\% \pm 0.3\%$ for Treg cells, respectively; Fig 7, A and B). LRBA-deficient Tconv but not Treg cells also exhibited increased Ki67 staining, which is indicative of increased proliferation (control vs LRBA-deficient subjects:

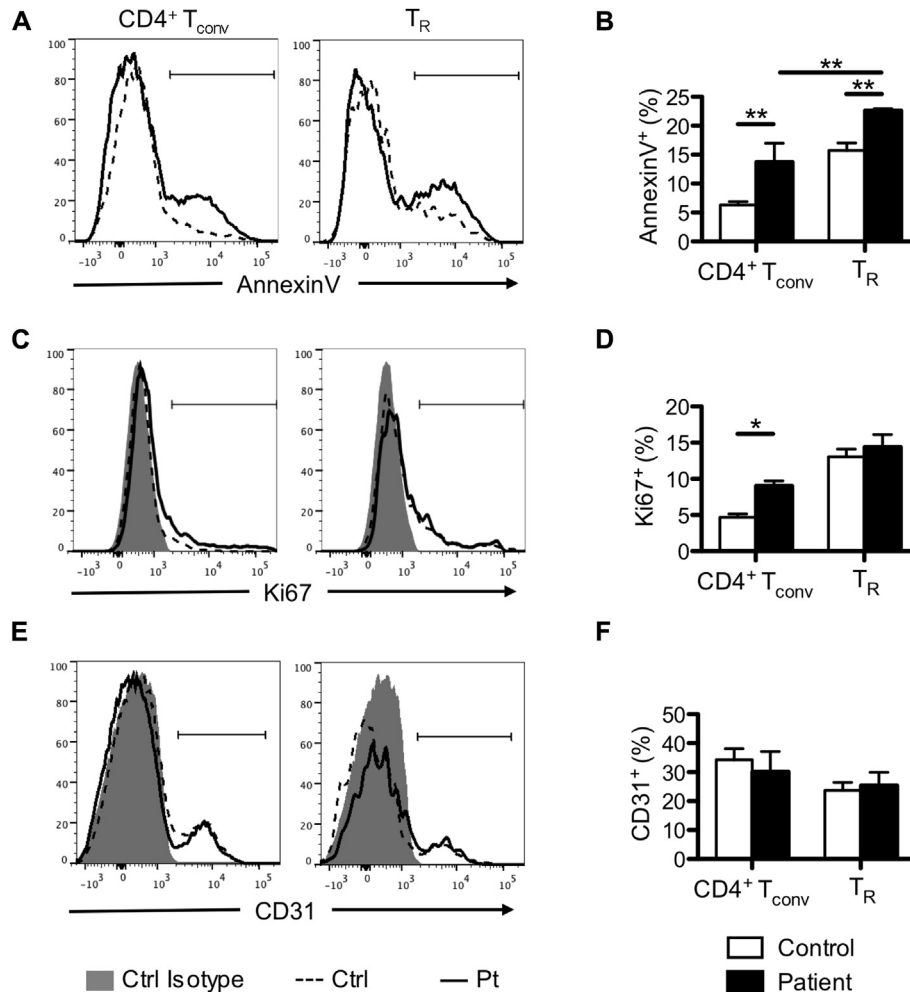


FIG 7. Increased turnover of LRBA-deficient T cells. **A, C, and E,** Flow cytometric analysis of Annexin V (Fig 7, A), Ki67 (Fig 7, C), and CD31 (Fig 7, E) staining in *ex vivo* CD4⁺ Tconv and Treg (T_R) cells from LRBA-deficient patients and control subjects. **B, D, and F,** Frequencies of Annexin V⁺ (Fig 7, B), Ki67⁺ (Fig 7, D), and CD31⁺ (Fig 7, F) cells within Tconv and Treg cell populations of LRBA-deficient patients and control subjects. There were 4 to 5 patients and 5 to 8 control subjects per group. **P* < .05 and ***P* < .01, 2-way ANOVA with posttest analysis.

4.7% ± 0.5% vs 9% ± 0.7% for CD4⁺ Tconv cells and 13% ± 1% vs 14.4% ± 1.7% for Treg cells, respectively; Fig 7, C and D). In contrast, the frequency of CD31⁺ T cells, a marker of recent thymic emigrant cells, was similar in peripheral blood of patients and control subjects (Fig 7, E and F). These results pointed to increased turnover (proliferation and apoptosis) of Tconv cells and increased attrition (apoptosis) of Treg cells in the periphery of LRBA-deficient patients in the face of apparently normal thymic efflux of the respective population.

The increased apoptosis in peripheral LRBA-deficient T cells prompted us to examine the activity of the metabolic sensors mammalian target of rapamycin complex (mTORC) 1 and 2, respectively. In particular, mTORC1 deficiency in Treg cells precipitates an IPEX-like phenotype in experimental mice, which is indicative of the essential role this pathway plays in regulating Treg cell metabolism.³³ Stimulation of PBMCs of LRBA-deficient patients and control subjects with anti-CD2/CD3/CD28 mAbs revealed that phosphorylation of S6 and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), downstream substrates of mTORC1, was decreased

in Treg and Tconv cells from patients versus control subjects (Fig 8, A-D).³³ Furthermore, phosphorylation of the kinase AKT at S473 residue (pAKTS473), a measure of mTORC2 kinase activity, was also decreased in Treg and Tconv cells from patients versus control subjects (Fig 8, E and F).³³ These findings indicated that LRBA deficiency was associated with impaired activation of mTORC1 and mTORC2, with a potentially deleterious effect on Treg cell function.

DISCUSSION

We demonstrate that LRBA deficiency results in decreased frequency, aberrant phenotype, and decreased suppressive function of Treg cells, leading in at least 1 case to an IPEX-like condition similar in severity to classical IPEX syndrome. A compromised Treg cell compartment is thus a cardinal feature of LRBA deficiency and might play a critical role in the ubiquitous autoimmune manifestations of the disease.

LRBA is a widely expressed multidomain protein that shares a highly conserved BEACH domain with other BEACH family

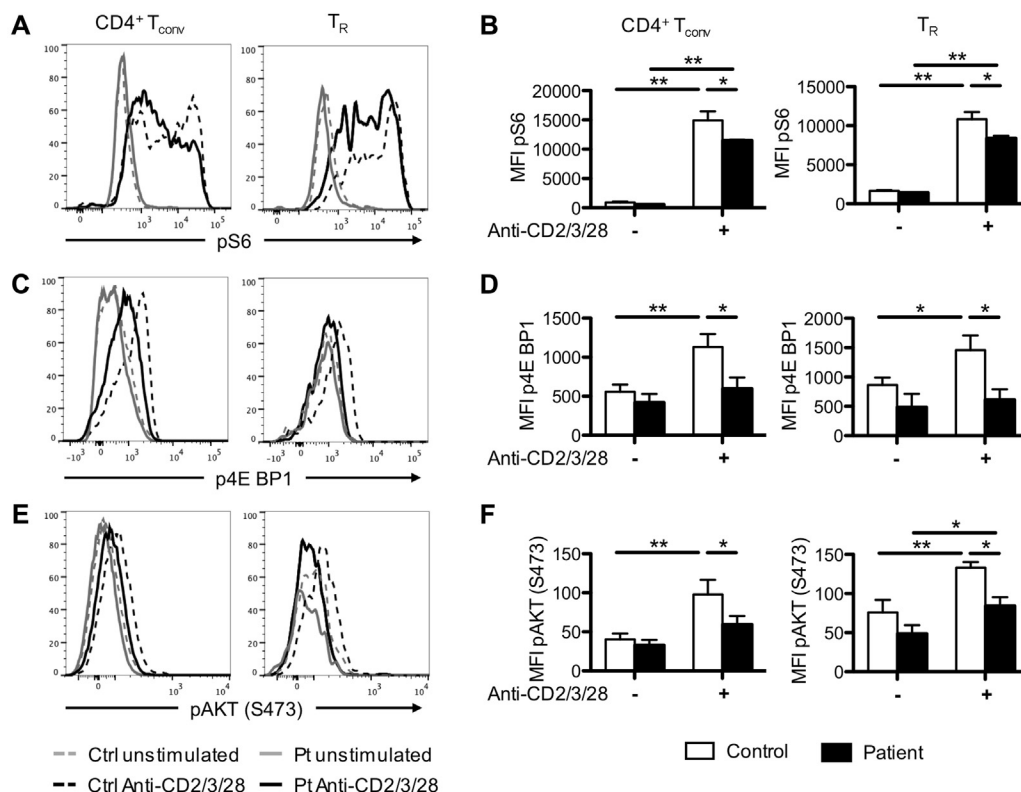


FIG 8. Impaired mTORC1 and mTORC2 activities in LRBA-deficient Tconv and Treg (T_R) cells. **A**, Flow cytometric analysis of pS6 of Tconv and Treg T cells from LRBA-deficient patients and control subjects before and after anti-CD2/CD3/CD28 stimulation. **B**, Mean fluorescence intensity (MFI) of pS6 within Tconv and Treg cells of LRBA-deficient patients and control subjects before and after anti-CD2/CD3/CD28 stimulation. **C**, Flow cytometric analysis of p4E-BP1 of Tconv and Treg cells from LRBA-deficient patients and control subjects after anti-CD2/CD3/CD28 stimulation. **D**, MFI of p4E-BP1 within Tconv and Treg cells of LRBA-deficient patients and control subjects before and after anti-CD2/CD3/CD28 stimulation. **E**, Flow cytometric analysis of p-AKT (S473) of Tconv and Treg cells from LRBA-deficient patients and control subjects after anti-CD2/CD3/CD28 stimulation. **F**, MFI of p-AKT (S473) within Tconv and Treg cells of LRBA-deficient patients and control subjects before and after anti-CD2/CD3/CD28 stimulation. * $P < .05$ and ** $P < .01$, 2-way ANOVA with posttest analysis.

proteins, including LYST, which is encoded by the Chediak-Higashi syndrome gene (*CHS1*).³⁴ It is highly conserved from *Homo sapiens* down to *Caenorhabditis elegans*, particularly in the functional domains, and has been implicated in regulating endosomal trafficking, cell proliferation, and survival.^{35,36} LRBA deficiency did not affect the *in vitro* differentiation of iTreg cells nor did it affect the fraction of recent thymic immigrants among peripheral Tconv and Treg cells. Instead, it was associated with increased apoptosis of Treg and Tconv cells, with increased turnover of the latter but not the former. A disproportionately constrained Treg cell population, especially in the context of a lymphopenic environment, has been invoked as a trigger for the development of autoimmune lymphoproliferation related to a severely restricted Treg cell T-cell receptor repertoire.³⁷

Treg cell depletion in patients with LRBA deficiency is compounded by impaired Treg cell phenotype and function, with decreased expression of key effector proteins involved in Treg cell suppression, such as CD25 and CTLA-4.³⁸ LRBA localizes to cytosol at lysosomes, the trans-Golgi network, the endoplasmic reticulum, the perinuclear endoplasmic reticulum, and endocytic vacuoles.³⁹ Thus it is spatially segregated from FOXP3, the expression of which is exclusively nuclear.⁷ In

searching for a mechanism that accounts for the adverse effect of LRBA deficiency on Treg cells, the finding of impaired activation of mTOR complexes invoked deranged metabolic sensing in disease pathogenesis.⁴⁰ In particular, defective mTORC1 cell activation might provide one mechanism by which LRBA deficiency impairs Treg cell function. In mice, Treg cell-specific mTORC1 deficiency, effected through deletion of the gene encoding the essential mTORC1 component Raptor, precipitates a Foxp3 deficiency-like disease, with runting, lymphoproliferation, and autoimmunity.³³ mTORC1 directs Treg cell lipid metabolism, which is relevant to their energy generation and suppressor function.³³ It affects the expression of several Treg cell markers, most notably CTLA-4, which can contribute to immune dysregulation on mTORC1 deficiency.³³

Mechanisms by which LRBA deficiency impairs mTOR activation can involve defective autophagy, as originally described by Lopez-Herrera et al.¹⁷ Autophagy regulates energy metabolism in T cells, and its dysfunction might result in decreased activation of mTOR complexes and attenuated phosphorylation of downstream targets, including S6 and 4E-BP1.^{41,42} Alternatively, LRBA might act as a scaffold that enables the assembly and activation of mTOR complexes,

recruitment of downstream intermediates (eg, AKT), or both, as has been described for other scaffold proteins.⁴³

LRBA deficiency is associated with intense autoantibody responses, even in the context of B-cell lymphopenia and hypogammaglobulinemia. Our studies have identified increased T_{FH} and decreased T_{FR} cell frequencies in the peripheral blood of LRBA-deficient subjects, which is consistent with ineffective regulation of antibody responses. T_{FR} cell deficiency and its associated T_{FH} cell dysregulation have been implicated in the development of autoantibodies in patients with immune dysregulatory diseases, such as IPEX syndrome.²⁵⁻²⁹ We hypothesize that this abnormality is a key mechanism in the development of humoral autoimmunity in patients with LRBA deficiency. Although the precise cause of T_{FH} dysregulation remains to be mapped, enhanced FOXO1/3a-induced activation of BCL6 because of decreased negative regulation of the former by pAKTS473 might provide one putative mechanism.⁴⁴ Further studies would be required to validate such a link.

Key messages

- LRBA deficiency results in profound Treg cell deficiency and impaired phenotype and function.
- Patients with LRBA deficiency may present with an IPEX-like disease, reflecting their profound Treg cell deficiency.
- LRBA-deficient subjects exhibit a high frequency of memory T cells and a dysregulated T_{FH} cell compartment with increased autoantibody production.

REFERENCES

- Verbsky JW, Chatila TA. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) and IPEX-related disorders: an evolving web of heritable autoimmune diseases. *Curr Opin Pediatr* 2013;25:708-14.
- Chatila TA, Blaaser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J Clin Invest* 2000;106:R75-81.
- Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 2001;27:20-1.
- Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 2001;27:18-20.
- Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked: forkhead box protein 3 mutations and lack of regulatory T cells. *J Allergy Clin Immunol* 2007;120:744-52.
- Barzaghi F, Passerini L, Bacchetta R. Immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome: a paradigm of immunodeficiency with autoimmunity. *Front Immunol* 2012;3:211.
- Lin W, Haribhai D, Relland LM, Truong N, Carlson MR, Williams CB, et al. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol* 2007;8:359-68.
- Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 2007;445:771-5.
- Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 2012;30:531-64.
- Hill JA, Feuerer M, Tash K, Haxhinasto S, Perez J, Melamed R, et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 2007;27:786-800.
- Chatila T. The regulatory T cell transcriptome: E pluribus unum. *Immunity* 2007;27:693-5.
- Barzaghi F, Passerini L, Gambineri E, Ciullini Mannurita S, Cornu T, Kang ES, et al. Demethylation analysis of the FOXP3 locus shows quantitative defects of regulatory T cells in IPEX-like syndrome. *J Autoimmun* 2012;38:49-58.
- Caudy AA, Reddy ST, Chatila T, Atkinson JP, Verbsky JW. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. *J Allergy Clin Immunol* 2007;119:482-7.
- Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. *Proc Natl Acad Sci U S A* 1997;94:3168-71.
- Cohen AC, Nadeau KC, Tu W, Hwa V, Dionis K, Bezrodnik L, et al. Cutting edge: Decreased accumulation and regulatory function of CD4+ CD25(high) T cells in human STAT5b deficiency. *J Immunol* 2006;177:2770-4.
- Sampaio EP, Bax HI, Hsu AP, Kristosturyan E, Pechacek J, Chandrasekaran P, et al. A novel STAT1 mutation associated with disseminated mycobacterial disease. *J Clin Immunol* 2012;32:681-9.
- Lopez-Herrera G, Tampella G, Pan-Hammarstrom Q, Herholz P, Trujillo-Vargas CM, Phadwal K, et al. Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. *Am J Hum Genet* 2012;90:986-1001.
- Alangari A, Alsultan A, Adly N, Massaad MJ, Kiani IS, Aljebreen A, et al. LPS-responsive beige-like anchor (LRBA) gene mutation in a family with inflammatory bowel disease and combined immunodeficiency. *J Allergy Clin Immunol* 2012;130:481-8.e2.
- Burns SO, Zenner HL, Plagnol V, Curtis J, Mok K, Eisenhut M, et al. LRBA gene deletion in a patient presenting with autoimmunity without hypogammaglobulinemia. *J Allergy Clin Immunol* 2012;130:1428-32.
- Ohsumi TK, Borowsky ML. MolBioLib: a C++11 framework for rapid development and deployment of bioinformatics tasks. *Bioinformatics* 2012;28:2412-6.
- Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, del Angel G, Levy-Moonshine A, et al. From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline. *Curr Protoc Bioinformatics* 2013;43:11.0.1-11.0.33.
- Engelhardt KR, McGhee S, Winkler S, Sassi A, Woellner C, Lopez-Herrera G, et al. Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. *J Allergy Clin Immunol* 2009;124:1289-302.e4.
- Li QZ, Zhou J, Wandstrat AE, Carr-Johnson F, Branch V, Karp DR, et al. Protein array autoantibody profiles for insights into systemic lupus erythematosus and incomplete lupus syndromes. *Clin Exp Immunol* 2007;147:60-70.
- Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 2008;28:546-58.
- Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* 2011;17:983-8.
- Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* 2011;17:975-82.
- Sage PT, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* 2013;14:152-61.
- Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* 2011;187:4553-60.
- Kinnunen T, Chamberlain N, Morbach H, Choi J, Kim S, Craft J, et al. Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. *Blood* 2013;121:1595-603.
- Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 2011;34:108-21.
- Saito R, Onodera H, Tago H, Suzuki Y, Shimizu M, Matsumura Y, et al. Altered expression of chemokine receptor CXCR5 on T cells of myasthenia gravis patients. *J Neuroimmunol* 2005;170:172-8.
- Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* 2010;62:234-44.
- Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature* 2013;499:485-90.
- Cullinane AR, Schaffer AA, Huizinga M. The BEACH is hot: a LYST of emerging roles for BEACH-domain containing proteins in human disease. *Traffic* 2013;14:749-66.
- de Souza N, Vallier LG, Fares H, Greenwald I. SEL-2, the *C. elegans* neurobeachin/LRBA homolog, is a negative regulator of lin-12/Notch activity

- and affects endosomal traffic in polarized epithelial cells. *Development* 2007;134:691-702.
36. Wang JW, Gamsby JJ, Highfill SL, Mora LB, Bloom GC, Yeatman TJ, et al. Deregulated expression of LRBA facilitates cancer cell growth. *Oncogene* 2004; 23:4089-97.
 37. Haribhai D, Williams JB, Jia S, Nickerson D, Schmitt EG, Edwards B, et al. A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity* 2011;35:109-22.
 38. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 2009;30:636-45.
 39. Wang JW, Howson J, Haller E, Kerr WG. Identification of a novel lipopolysaccharide-inducible gene with key features of both A kinase anchor proteins and chs1/beige proteins. *J Immunol* 2001;166:4586-95.
 40. Dunlop EA, Tee AR. mTOR and autophagy: a dynamic relationship governed by nutrients and energy. *Semin Cell Dev Biol* 2014 [Epub ahead of print]. <http://dx.doi.org/10.1016/j.semcdb.2014.08.006>.
 41. Hubbard VM, Valdor R, Patel B, Singh R, Cuervo AM, Macian F. Macroautophagy regulates energy metabolism during effector T cell activation. *J Immunol* 2010; 185:7349-57.
 42. Kang HT, Lee KB, Kim SY, Choi HR, Park SC. Autophagy impairment induces premature senescence in primary human fibroblasts. *PLoS One* 2011;6:e23367.
 43. Fritzius T, Moelling K. Akt- and Foxo1-interacting WD-repeat-FYVE protein promotes adipogenesis. *EMBO J* 2008;27:1399-410.
 44. Hurtz C, Hatzi K, Cerchietti L, Braig M, Park E, Kim YM, et al. BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia. *J Exp Med* 2011;208:2163-74.

CASE REPORTS

Patient P2 (family B) is a 17-year-old boy with a diagnosis of CVID and granulomatous disease who has been receiving monthly intravenous immunoglobulin (IVIG) infusions since the age of 7 years. He initially presented at 7 years of age with a history of recurrent upper respiratory tract infections and chronic sinusitis. His weight and height were less than the 5th percentile. Initially, he had low WBCs and low absolute neutrophil counts. At 10 years of age, he had recurrent fevers and splenomegaly. The latter has persisted with a splenic size of greater than 20 cm in length. Computed tomographic (CT) scans of the chest, abdomen, and pelvis showed numerous enlarged matted lymph nodes in the right paratracheal and subcarinal regions, along with splenomegaly and enlarged left para-aortic lymph nodes. Lymph node biopsy specimens revealed follicular and sinus hyperplasia, frequent sinus histiocytes demonstrating erythrophagocytosis, and occasional small noncaseating granulomas without giant cells. Mycobacterial culture results were negative. He had frequent hospital admissions for recurrent pneumonia and IVIG infusions.

At age 11 years he was admitted to the hospital in severe respiratory distress. A CT scan revealed persistent enlarged lymphadenopathy, progression in the extent of nodular infiltration with air bronchograms, and consolidations in the upper lobes. A lung biopsy specimen displayed severe acute and chronic inflammation with granulation tissue and fibrosing pneumonitis. An axillary lymph node biopsy revealed acute lymphadenitis and noncaseating granulomas. He was given a diagnosis of sarcoidosis-like granulomas in the setting of CVID. He received a course of steroids with minimal improvement. Because of the progression of his pulmonary disease, he was started on

infliximab infusions. He has been clinically stable since receiving monthly infusions of IVIG and infliximab and daily oral azathioprine.

Patient P3, the sister of P2, is a 14-year-old girl who was born after a full-term pregnancy with no perinatal complications. She was given a diagnosis of biliary atresia shortly after birth and successfully underwent a Kasai procedure at the age of 2 months, with normalization of her liver enzyme levels by 2½ years of age. At age 10 years, she had hepatosplenomegaly with increases in liver enzyme levels. She also started experiencing recurrent upper respiratory tract infections, including sinusitis and mastoiditis. At 13 years of age, she became progressively fatigued and had increased hepatosplenomegaly and generalized lymphadenopathy, as determined based on CT scans. She also had new-onset pancytopenia (absolute neutrophil count, 200-700 cell/ μ L; platelets, 50,000-100,000/ μ L). Evaluation revealed evidence of chronic liver disease with signs of portal hypertension. CT studies revealed innumerable bilateral lung nodules and widespread lymphadenopathy in the neck, chest, and abdomen. She was started on steroids and IVIG and continued to receive monthly infusions of IVIG for maintenance.

METHODS

Antibodies

Anti-human mAbs to the following were used for staining: CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD127 (A019D5), CD25 (BC96), CD31 (WM59), CD45RO (UCHL1), CD45RA (HI100), CCR7 (G043H7, BioLegend), CTLA4 (14D3), CXCR5 (MU5UBEE), FOXP3 (PCH101), Helios (22F6), PD-1 (eBioJ105), CD40L (24-31), ICOS (D10.G4.1), Bcl6 (BCL-UP), Ki67 (20Raj1), phospho-S6 ribosomal (cupk43k), phospho-4E-BP1 (V3NTY24, eBioscience), pAKT (S473; D9E; cell signaling), and the appropriate isotype controls.

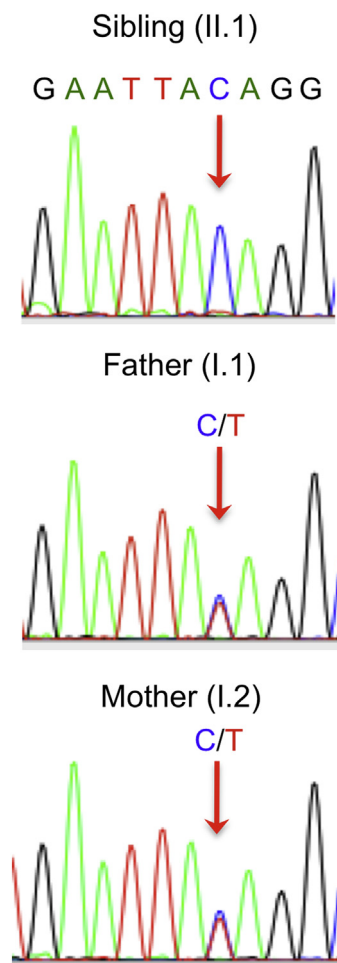


FIG E1. Sanger sequence analysis of genomic DNA of members of family A at the site of the *LRBA* mutation in patient P1. The Roman and Arabic numeral designations correspond to the same numerals in the pedigree of family A in Fig 1. Arrows represent the site of the nucleotide substitution (C→T). The sibling shown (II.1) had a normal sequence, whereas the parents showed a heterozygous pattern.

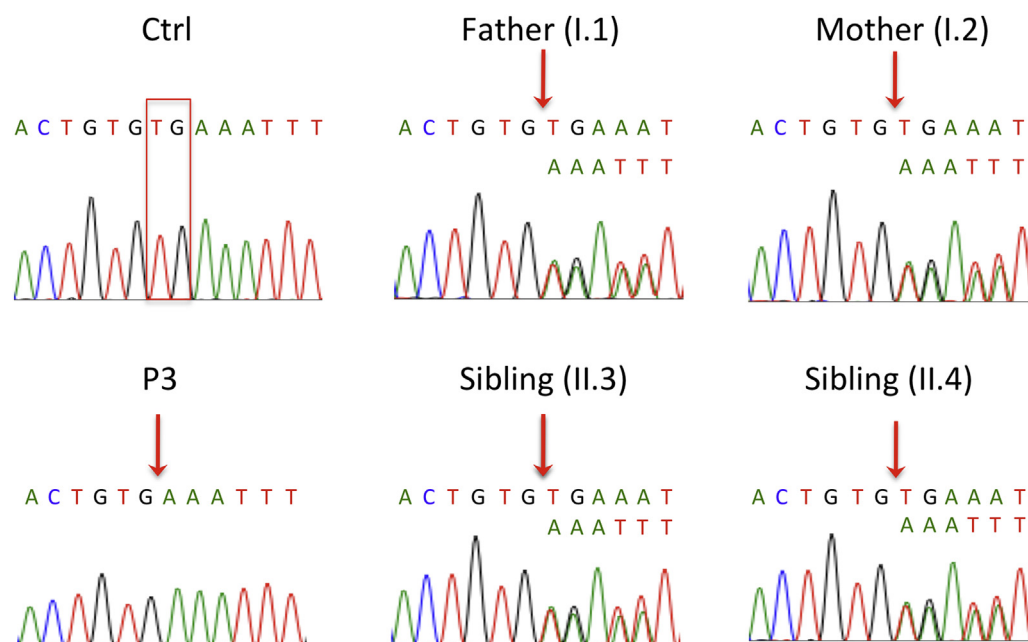


FIG E2. Sanger sequencing analysis of genomic DNA of members of family B at the site of the *LRBA* mutation in patients P2 and P3. The Roman and Arabic numeral designations correspond to the same numerals in the pedigree of family B in Fig 1. The deleted TG nucleotides are delineated by a red box in the control sequence, whereas the red arrows represent the site of the deletion in the respective subject. All clinically unaffected members of the family were found to be heterozygous for the TG deletion. Their upper sequence is that of the normal allele, whereas the lower sequence is the mutant allele.

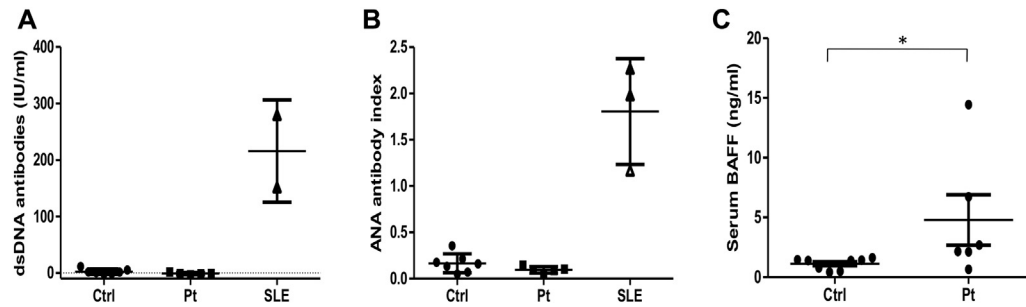


FIG E3. Serum levels of double-stranded DNA (*dsDNA*) antibodies (**A**), anti-nuclear antibodies (*ANA*; **B**), and serum B-cell activation factor (*BAFF*; **C**) in control subjects (*Ctrl*), LRBA-deficient patients (*Pt*), and patients with SLE. * $P < .05$.

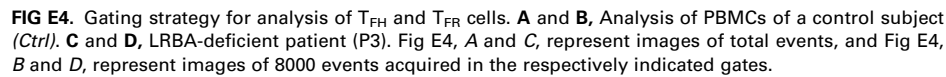


FIG E4. Gating strategy for analysis of T_{FH} and T_{FR} cells. **A** and **B**, Analysis of PBMCs of a control subject (*Ctrl*). **C** and **D**, LRBA-deficient patient (P3). Fig E4, **A** and **C**, represent images of total events, and Fig E4, **B** and **D**, represent images of 8000 events acquired in the respectively indicated gates.

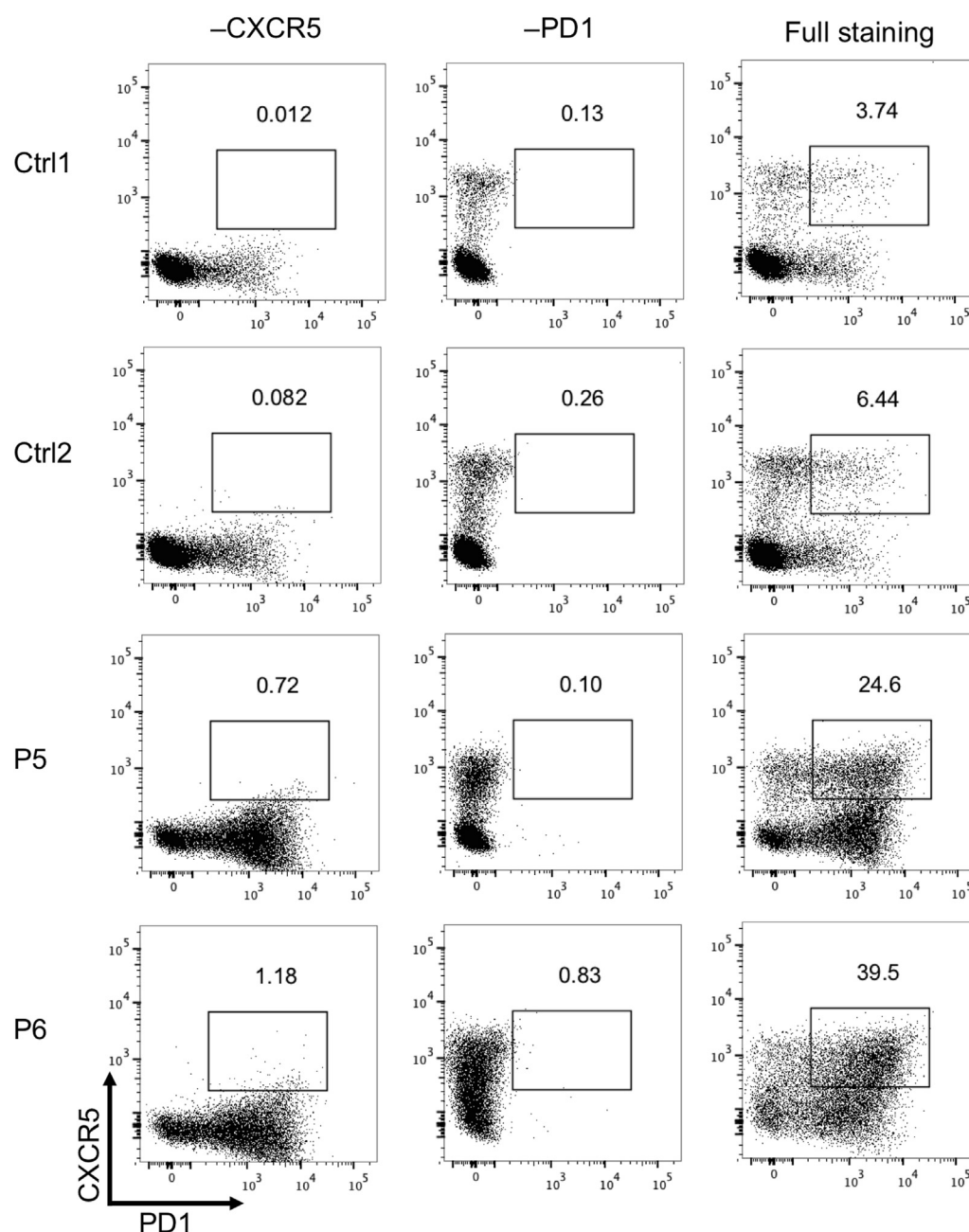


FIG E5. Representative fluorescence minus one analyses of circulating T_{FH} populations in patients and control subjects. PBMCs were stained with a cocktail of mAbs against CD3, CD4, and PD-1 but not CXCR5 (left column); CD3, CD4, and CXCR5 but not PD-1 (middle column); or all 4 antigens (right column). Numbers with the flow panels indicate percentage cells within the T_{FH} gate.

TABLE E1. Hematologic and immunologic findings on LRBA-deficient patients of families A and B

	Patient P1	Patient P2	Patient P3
Sex; age (y)	Male; 2½	Male; 17	Female; 14
Peripheral blood analysis			
WBC (cells/ μ L)	8,900	4,700	1,200
Hemoglobin (g/dL)	12.7	10.6	10.9
Platelets (cells/ μ L)	340,000	136,000	80,000
Neutrophils (cells/ μ L)	4,010 (45%)	3,008 (64%)	288 (24%)
Lymphocytes (cells/ μ L)	2,920 (33%)	1,279 (27%)	756 (63%)
Monocytes (cells/ μ L)	430 (4.8%)	282 (6%)	120 (10%)
Eosinophils (cells/ μ L)	1,140 (12.8%)	141 (3%)	36 (3%)
Lymphocyte analysis			
CD3 ⁺ (cells/ μ L)	1,787 (62%)	916 (94%)	525 (84%)
CD3 ⁺ /CD4 ⁺ (cells/ μ L)	951 (33%)	681 (70%)	362 (58%)
CD3 ⁺ /CD8 ⁺ (cells/ μ L)	577 (20%)	193 (20%)	135 (22%)
CD19 ⁺ (cells/ μ L)	950 (33%)	11 (1%)	50 (8%)
CD16 ⁺ /CD56 ⁺ (cells/ μ L)	115 (4%)	43 (4%)	49 (8%)
CD4/CD8 ratio	1.65	3.53	2.67
CD3 ⁺ /CD4 ⁺ /CD45RA ⁺	46%	Not done	Not done
CD3 ⁺ /CD4 ⁺ /CD45RO ⁺	64%	Not done	Not done
Immunoglobulins (age)	(2½ y)	(7 y)	(13 y)
IgG (mg/dL)	929	212	891
IgA (mg/dL)	93	<22	78
IgM (mg/dL)	34	<17	213
IgE (IU/mL)	<25	<2	4.14
Tetanus titer (IU/mL)	0.64 (protective)	Not detectable	0.25 (immunity present; requires booster)
Pneumococcal titer (μ g/mL)	>270 (protective)	Not done	18.6 (protective)
Diphtheria titer (IU/mL)	0.2 (immunity present; requires booster)	Not done	0.08 (not protective)

The age at which the immunoglobulin determination is reported for the respective patient is either the most recent one available (P1) or the one just before the start of IVIG therapy (P2 and P3).

TABLE E2. List of nonsynonymous candidate variants identified by using WES in patient P1

Chromosome	Starting coordinate	Reference sequence	Patient sequence	Gene
chr01	1356358	—	CTCCGC	<i>ANKRD65</i>
chr01	13497572	G	A	<i>PRAMEF16</i>
chr01	13497584	C	T	<i>PRAMEF16</i>
chr01	16558757	C	T	<i>RSG1</i>
chr01	54605319	—	CG	<i>CDCP2</i>
chr04	151817581	G	A	<i>LRBA</i>
chr05	40843764	C	T	<i>CARD6</i>
chr07	141721534	T	C	<i>MGAM</i>
chr08	144775915	C	T	<i>ZNF707</i>
chr08	144885665	C	T	<i>SCRIB</i>
chr08	144945438	G	A	<i>EPPK1</i>
chr09	40705431	G	A	<i>FAM75A3</i>
chr12	11183451	A	C	<i>TAS2R31</i>
chr12	11183451	A	C	<i>TAS2R31</i>
chr15	82637174	—	CCCTCTCC	<i>GOLGA6L10</i>
chr21	45978028	G	T	<i>KRTAP10-3</i>
chr22	17265124	A	T	<i>XKR3</i>
chrX	101908924	T	G	<i>GPRASP1</i>

The 18 candidate variants were homozygous in the patient, heterozygous in his mother, and either absent or heterozygous in his healthy sibling. They were absent from dbSNP and not present in the homozygous state in an in-house collection of 80 Middle Eastern exomes. The gene symbol (*LRBA*) for the candidate gene identified on chromosome 4 is in boldface.

TABLE E3. List of nonsynonymous candidate variants identified by using WES in patient P2

Chromosome	Starting coordinate	Reference sequence	Patient sequence	Gene
chr01	980658	G	T	AGRN
chr01	2460963	—	CGG	HES5
chr01	21978270	A	C	RAP1GAP
chr01	38077350	—	G	RSPO1
chr01	151811210	C	A	C2CD4D
chr01	152192409	C	T	HRNR
chr01	155247583	C	T	HCN3
chr01	159899764	T	A	IGSF9
chr02	220423154	G	T	OBSL1
chr04	151837581	CA	—	LRBA
chr04	164435295	G	T	C4orf43
chr07	4874318	G	A	RADIL
chr07	6732193	G	A	ZNF12
chr14	92537378	—	CTGCTG	ATXN3
chr16	81190601	T	A	PKD1L2
chr19	2807544	C	T	THOP1
chr19	2807748	G	A	THOP1
chr19	12902872	A	G	JUNB
chr19	55895198	C	A	TMEM238
chr20	62195445	G	A	PRIC285
chrX	14871169	C	T	FANCB
chrX	38019394	G	C	SRPX
chrX	38145633	TTCCTCCCCTTCTTC	—	RPGR
chrX	38147291	GTT	—	RPGR
chrX	53222960	T	C	KDM5C
chrX	65479994	A	C	HEPH
chrX	70146883	G	T	SLC7A3
chrX	118923946	C	T	RPL39

The 28 candidate variants were homozygous and nonsynonymous in the patient, absent from dbSNP, and not present in the homozygous state in our in-house collection of 80 Middle Eastern exomes. The gene symbol (*LRBA*) for the candidate gene identified on chromosome 4 is in boldface.