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Regulatory T Cell Deficiency and Immune dysregulation, Polyendocrinopathy, Enteropathy, X-Linked-Like Disorder Due to Loss of Function Mutations in *LRBA*

Louis-Marie Charbonnier, PhD^{a,*}, Erin Janssen, MD, PhD^{a,*}, Janet Chou, MD^a, Toshiro K. Ohsumi, PhD^b, Sevgi Keles, MD^a, Joyce T. Hsu, MD^a, Michel J. Massaad, PhD^a, Maria Garcia-Lloret, MD^c, Rima Hanna-Wakim, MD^d, Ghassan Dbaiibo, MD^d, Abdullah A. Alangari, MD^e, Abdulrahman Alsultan, MD^e, Daifulah Al-Zahrani, MD^f, Raif S. Geha, MD^{a,**}, and Talal A. Chatila, MD, MSc^{a,**}

^aDivision of Immunology, The Boston Children's Hospital. Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA

^bDepartment of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts, USA

^cDivision of Immunology, Department of Pediatrics, University of California at Los Angeles, Los Angeles, California USA

^dDivision of Pediatric Infectious Diseases, American University of Beirut, Beirut, Lebanon

^eDepartment of Pediatrics, College of Medicine, King Saud, University Saudi Arabia

^fImmunology and Allergy, Pediatric Department, King Abdulaziz Medical City-WR, Jeddah, Saudi Arabia

Abstract

Background—A number of heritable immune dysregulatory diseases result from defects affecting T regulatory (T_R) cell development and/or function. They include Immune dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX), due to mutations in *FOXP3*, and IPEX-like disorders caused by mutations in *IL2RA*, *STAT5b* and *STAT1*. However, the genetic defects underlying many cases of IPEX-like disorders remain unknown.

Objective—We sought to identify the genetic abnormalities in subjects with idiopathic IPEX-like disorders.

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Corresponding author; Talal A. Chatila, Division of Immunology, The Boston Children's Hospital, One Blackfan Circle, Boston, Massachusetts 02115, USA. Tel. (617) 919 3529; Fax: (617) 730 0528. talal.chatila@childrens.harvard.edu.

*These authors contributed equally to this work.

**These authors contributed equally to this work

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Methods—We performed whole exome and targeted gene sequencing, and phenotypic and functional analyses of T_R cells.

Results—A child who presented with an IPEX-like syndrome and severe T_R cell deficiency was found to harbor a nonsense mutation in the gene encoding LPS-responsive beige-like anchor (LRBA), previously implicated as cause of common variable immunodeficiency with autoimmunity. Analysis of subjects with LRBA deficiency revealed marked T_R cell depletion, profoundly decreased expression of canonical T_R cell markers, including FOXP3, CD25, Helios, and CTLA4 and impaired T_R 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 T_R cells were normal, LRBA-deficient T cells exhibited increased apoptosis and reduced activities of the metabolic sensors mammalian target of rapamycin 1 and 2 complexes.

Conclusion—LRBA deficiency is a novel cause of IPEX-like syndrome and T_R cell deficiency associated with metabolic dysfunction and increased apoptosis of T_R cells.

Keywords

Autoantibodies; Autoimmunity; FOXP3; IPEX; LRBA; mTOR; T Follicular Helper Cells; T Follicular Regulatory Cells; T Regulatory Cells

Introduction

A number of Mendelian disorders of immune dysregulation and autoimmunity result from defects affecting T regulatory (T_R) cell development and function¹. The best characterized of these is Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX), resulting from deleterious mutations affecting *FOXP3*^{2–4}. Subjects with IPEX suffer from a plethora of autoimmune manifestations, including enteropathy, endocrinopathies especially type 1 diabetes and thyroiditis, and cytopenias^{5, 6}. FOXP3 directs the differentiation of T_R cells and is essential for their suppressive functions^{7–9}. It reinforces and stabilizes transcriptional networks characteristic of T_R cells and maintains T_R cell phenotypic markers^{10, 11}. In the absence of a functional FOXP3 protein, the expression of these networks and markers is attenuated and/or disrupted.

In cohorts of subjects with an IPEX-like phenotype, up to half of the patients fail to exhibit mutations in *FOXP3*^{5, 12}. A number of other gene defects that affect T_R cell function give rise to an IPEX-like phenotype¹. These include loss of function mutations in genes encoding components of the interleukin 2 receptor pathway, including *IL2RA* and *STAT5b*, and gain of function mutations in *STAT1*^{1, 13–16}. Nevertheless, defects underlying the majority of cases with 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 T_R cell deficiency, and normal *FOXP3* gene sequence was found to have a homozygous nonsense mutation in the *LPS-responsive beige-like anchor (LRBA)* gene, previously implicated as a cause of common variable immunodeficiency (CVID) with

autoimmunity^{17–19}. Analysis of other subjects with LRBA deficiency also revealed T_R cell deficiency, profoundly decreased canonical T_R cell markers, and impaired T_R cell suppressive function. Patients with LRBA deficiency manifested elevated autoantibodies against autologous antigens in association with a dramatic increase in the frequency of circulating T follicular helper T (T_{FH}) cells and depressed circulating T follicular regulatory (T_{FR}) cells. Our findings implicate LRBA deficiency as a cause of IPEX-like syndrome and suggest that T_R cell dysfunction is a major contributor of the autoimmune manifestations in affected subjects.

Materials and Methods

Patients

Affected individuals with LRBA deficiency from 3 families were analyzed. The index cases of families A and B are described herein for the first time. Those of family C have been previously reported¹⁸. A patient with IPEX due to an A384T amino acid substitution in FOXP3 was identified by WES (M. Garcia-Lloret and T. A. Chatila; manuscript in preparation). Subjects with the diagnosis of SLE were identified at the Rheumatology clinic at the Boston children's Hospital. Control subjects were age group-matched. All study participants were recruited using written informed consent approved by the local Institutional Review Boards. Studies at the Boston Children's Hospital were conducted under approved protocol #04-09-113R.

Antibodies and flow cytometry

Information on the antibodies employed is provided in the Online Methods in the Repository. Whole blood was incubated with mAbs against surface markers for 30 min on ice. Intracellular staining with FOXP3, CTLA4, Helios, Bcl6 and CD40L mAbs was performed using eBioscience Fixation/Permeabilization according to the manufacturer's instructions. Intracellular staining for pS6, p4E-BP1 and pAKT (S473) were performed on freshly isolated peripheral blood mononuclear cells (PBMCs) either left untreated or stimulated with anti-CD2/3/28 mAb-coated beads (Miltenyi) for 4 or 12 hours using eBioscience Fixation/Permeabilization buffer. Annexin V (Biolegend) staining was performed on PBMCs using Biolegend Annexin V binding buffer according to the manufacturer's instructions. Data were collected with a Fortessa cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Whole exome sequencing (WES)

WES was performed on genomic DNA of the probands of families A and B, their parents, and their healthy siblings through Axseq, Inc. The Agilent SureSelect Target enrichment kit was used for exon capture (Agilent). Paired-end sequencing was performed using an Illumina HiSeq2000 instrument, which generated 100 base pair reads. Average coverage of the exome was 183–208x. Analysis of WES data was performed with MolBioLib²⁰. Variants were identified using Genome Analysis Toolkit with standard filtering parameters according to Genome Analysis Toolkit Best Practices Recommendations²¹. Variant calls with a read coverage $\geq 2\times$ and a Phred-scaled SNP quality ≥ 20 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 described ²². Immunoblots were carried out using polyclonal rabbit anti-LRBA antibodies (Sigma-Aldrich, St. Louis, MO). The blots were reprobed with mouse anti- β -actin mAb (Clone C4) (Millipore, Billerica, MA).

Regulatory T cell Suppression Assays

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

In vitro generation of induced regulatory T cells

CD4⁺ T cells were isolated from PBMCs by negative selection using magnetic beads (Miltenyi). Naïve CD3⁺CD4⁺CD25⁻ CD45RO⁻CD45RA⁺ cells were purified from CD4⁺ T cells by cell sorting on a FACS ARIA (purity > 98%). Naïve CD4 T cells were resuspended in complete media and seeded at a concentration of 5 $\times$ 10⁴/well of a 96-well plate and stimulated with anti-CD2/CD3/CD28 mAb-coated beads (Miltenyi) and 5ng/mL of recombinant human TGF- β 1 (R&D system). After 4 days, intracellular staining for FOXP3 was performed.

Cytokine and autoantibody assays

For IL-10 assays, cell-sorted T_R cells were seeded in round bottom wells at 5 $\times$ 10³ cells/well and either left untreated or treated with anti-CD2/CD3/CD28 mAb-coated beads or with PMA/ionomycin (20 ng/ml and 1 μ M, respectively). The supernatant were harvested 4 days later and analyzed for IL-10 by ELISA (eBioscience). For autoantibody detection, plasma aliquots from patient and control subjects were analyzed using microarrays spotted with 84 autoantigens (University of Texas Southwestern Medical Center, Genomic and Microarray Core Facility), as described ²³. Data was normalized to healthy controls. Anti-nuclear antigens (ANAs) and dsDNA (double stranded DNA) antibodies were measured by enzyme-linked immunosorbant assay (ELISA) (Genway Biotech and Alpha Diagnostics).

Statistical Analysis

Aggregate results are presented as means $\pm$ standard error of the means (S.E.M.).

Comparison between groups was carried out using Student's unpaired two tailed *t* test and 2-way ANOVA with Bonferroni post-test analysis, as indicated. Differences in mean values were considered significant at a $p < 0.05$.

Results

In family A (Figure 1 and Figure E1 in the Online Repository), the index case (P1) is a 2 year 10 month old Saudi Arabian boy who presented at the age of 3 months with protracted diarrhea following 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 diagnosed with type 1 diabetes with positive insulin antibodies and hypothyroidism with positive thyroid antibodies. He also developed a Coombs positive autoimmune hemolytic anemia. In addition, he suffered recurrent episodes of sepsis, including *Pseudomonas aeruginosa* septicemia and *Streptococcus pneumoniae*-related septic shock. Analysis of his immunological status revealed reduced T cell proliferative responses to phytohemagglutinin (~50% of normal), normal B cell numbers, and elevated immunoglobulin levels in the setting of low normal specific antibody responses (Table E1 in the Online Repository). Analysis of his *FOXP3* gene sequence failed to reveal the presence of deleterious mutations. In view of his consanguineous heritage, we undertook WES to identify gene variants which 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 non-synonymous, absent from dbSNP, and not present in the homozygous state in any of our 80 in-house Middle Eastern exomes (Table E2 in the Online Repository). While the majority of these variants scored benign by Polyphen and/or SIFT protein function prediction algorithms, the one variant that stood out in relation to its deleterious impact on the immune system involved *LRBA*^{17–19}. The patient suffered 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 in absent protein expression (Fig. 1A-D). The mutation was confirmed by Sanger sequencing, and both parents were heterozygous carriers of the mutation (Figure E1 in the Online Repository).

LRBA deficiency was also found in a second newly described Lebanese family with two siblings (Family B; P2 and P3), whose clinical histories were notable for recurrent infections and immune dysregulation (Supplementary Text in the Online Repository). A homozygous exonic deletion in *LRBA* (c.865_866del) was the lead candidate variant identified by WES under the aforementioned filtering conditions (Table E3 in the Online Repository). It was confirmed by Sanger sequencing, and resulted in absent protein expression (Figure 1A-D). Both parents and the patients' two clinically unaffected siblings (II.3, and II.4; Figure 1A) were heterozygous carriers of the mutation (Figure E2 in the Online Repository). We also studied three previously described Saudi Arabian siblings (P4, P5 and P6; Family C) with LRBA deficiency due to a homozygous deletion in the BEACH domain of *LRBA* that

abolished protein expression (Figure 1A, B, D)¹⁸. The clinical and laboratory findings of these patients are detailed in Table E1 in the Online Repository.

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

We further analyzed the impact of LRBA deficiency on T_R cell suppressive function using an *in vitro* suppression assay of T cell proliferation to mitogenic stimulation. T_R cells were isolated by cell sorting of CD4⁺CD25⁺CD127^{low} T_R cells. They were confirmed by intracellular staining to be >90% positive for FOXP3, indicative of their T_R cell lineage (data not shown). Equal numbers of patient and control T_R cells, were added to an equal number of control CD4⁺CD25⁻ T_{eff} cells loaded with the proliferation dye CellTrace Violet and treated with a mitogenic combination of CD2/CD3/CD28 mAbs. LRBA-deficient T_R cells manifested decreased suppression of T cell proliferation, measured by tracer dye dilution in target T_{eff} cells, as compared to control T_R cells, indicative of their impaired function (Figure 3A, B). Defective *in vitro* suppression by LRBA-deficient T_R cells could not be ascribed to decreased IL-10 production, whose deficiency in T_R cells is associated with colitis²⁴. IL-10 production was increased in patient as compared to control T_R cells (Figure 3C).

T_R cell deficiency is associated with dysregulated T cell activation and skewing towards a memory phenotype. Consistent with immune dysregulation, LRBA deficiency resulted in skewing of peripheral CD4 and CD8 T cell towards a memory (CD45RA⁻CD45RO⁺) phenotype (control vs. LRBA deficient subjects: 59.5%±2.8 vs. 86%±2.1 for CD4 T cells, and 29.4%±4.2 vs. 50.3%±6.2 for CD8 T cells, respectively) (Figure 4A-D). The frequency of naïve CD45RA⁺CCR7⁺ CD8⁺T cells was decreased in LRBA-deficient subjects (control vs. LRBA deficient subjects: 10.4%±3 vs. 3%±0.7). However, there was no difference in the frequency of CD8⁺CD45RA⁺CCR7⁻ [(CD45RA⁺ effector memory (TEMRA)] T cells as compared to control subjects (Figure 4E-F).

LRBA-deficient subjects suffer from diverse autoimmune phenomena, including autoimmune hemolytic anemia, thrombocytopenia, thyroiditis, and end organ inflammation including colitis and pneumonitis¹⁷⁻¹⁹. Using an array of 84 autoantigens, we determined the spectrum of circulating autoantibodies in the 6 LRBA deficient patients as compared to an IPEX patient with a missense mutation in forkhead domain of FOXP3 (p.A384T), an adolescent patient with systemic lupus erythematosus (SLE), and five 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 subject with true IPEX disease (Figure 5A and data not shown). There was a predominance of cytoplasmic- and RNA-directed autoantibodies in LRBA deficient patients. Similar to IPEX patients and unlike SLE patients, they were negative for anti-nuclear and anti-dsDNA antibodies (Figure 5A; Figure E3 in the Online Repository and data not shown).

A specialized subset of T_R cells, termed T follicular regulatory (T_{FR}) cells, regulates the ability of T follicular helper (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 IPEX patients and in 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_{FH} ($CD4^+FOXP3^-CXCR5^+PD-1^{hi}$) 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 human diseases associated with intense autoantibody production such as SLE, myasthenia gravis, and juvenile dermatomyositis^{30–32}. The gating strategy for detecting circulating T_{FH} and T_{FR} cells and the specificity of the staining are illustrated in Figures E4 and E5 in the Online Repository, respectively. There was a striking increase in the frequency of T_{FH} cells in the peripheral blood of LRBA-deficient patients (controls: $4.9\% \pm 0.6$ vs. patients: $31.2\% \pm 5.3$) (Figure 5B, C). The patient CXCR5⁺PD-1^{hi} cells expressed high levels of B cell lymphoma 6 protein (BCL6), inducible co-stimulator protein (ICOS) and CD40 ligand (CD40L), consistent with their T_{FH} phenotype (Figure 5D). Within the T_{FH} cell population, the frequency of FOXP3⁺ T_{FR} cells was significantly decreased in patients relative to controls (controls: $10.3\% \pm 2$ vs. patients: $3.5\% \pm 0.6$) (Figure 5E, F). These results are consistent with dysregulated T_{FH} and compromised T_{FR} cell compartments in LRBA-deficient subjects.

To determine if LRBA deficiency impaired the induction of FOXP3 expression, we analyzed the capacity of LRBA-sufficient and deficient naïve $CD4^+$ T cells to differentiate into induced (i) T_R cells upon stimulation with anti-CD2/CD3/CD28 mAbs in presence of TGF-beta. $CD4^+$ T cells of patient and control subjects were enriched by magnetic separation, and naïve $CD4^+$ T cells were purified >98% purity by cell sorting (Figure 6A and data not shown). LRBA sufficient and deficient T cells were found equivalent in their capacity to differentiate into i T_R cells that equally expressed high levels of FOXP3 (Figure 6B, C).

Given the normal differentiation of LRBA-deficient T cells into i T_R cells, we examined the turnover of peripheral blood T_R and $CD4^+$ conventional T (T_{conv}) cells in patient vs. control subjects. Results revealed that both T_R and T_{conv} cells of LRBA-deficient subjects exhibited increased AnnexinV staining as compared to those of controls, indicative of increased apoptosis (control vs. LRBA deficient subjects: $6.3\% \pm 0.6$ vs. $13.7\% \pm 3.2$ for $CD4^+$ T_{conv} cells and $15.7\% \pm 1.3$ vs. $22.6\% \pm 0.3$ for T_R cells, respectively) (Figure 7A, B). LRBA-deficient T_{conv} but not T_R cells also exhibited increased Ki67 staining, indicative of increased proliferation (control vs. LRBA deficient subjects: $4.7\% \pm 0.5$ vs. $9\% \pm 0.7$ for $CD4^+$ T_{conv} cells and $13\% \pm 1$ vs. $14.4\% \pm 1.7$ for T_R cells, respectively) (Figure 7C, D). In contrast,

the frequency of CD31⁺ T cells, a marker of recent thymic emigrant cells (RTEs), was similar in peripheral blood of patient and control subjects (Figure 7E, F). These results pointed to increased turnover (proliferation and apoptosis) of T_{conv} cells and increased attrition (apoptosis) of T_R cells in the periphery of LRBA-deficient subjects in the face of apparently normal thymic efflux of the respective population.

The increased apoptosis in peripheral LRBA-deficient T cells promoted us to examine the activity of the metabolic sensors mammalian target of rapamycin 1 and 2 complexes (mTORC1 and mTORC2, respectively). In particular, mTORC1 deficiency in T_R cells precipitates an IPEX like phenotype in experimental mice, indicative of the essential role this pathway plays in regulating T_R cell metabolism³³. Stimulation of PBMCs of LRBA-deficient 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 patient vs. control T_R and T_{conv} cells (Figure 8A-D)³³. Furthermore, phosphorylation of the kinase AKT at S473 residue (pAKTS473), a measure of mTORC2 kinase activity, was also decreased in patient vs. control T_R and T_{conv} cells (Figure 8E-F)³³. These findings indicated that LRBA deficiency was associated with impaired activation of mTORC1 and mTORC2 complexes, with potentially deleterious impact on T_R cell function.

Discussion

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

LRBA is widely expressed multi-domain protein that shares a highly conserved beige and Chediak-Higashi syndrome (BEACH) domain with other BEACH family proteins including LYST, encoded by the Chediak-Higashi syndrome gene (*CHS1*)³⁴. It is highly conserved from *Homo sapiens* down to *C. elegans*, particularly in the functional domains, and has been implicated in regulating endosomal trafficking, cell proliferation and survival^{35, 36}. LRBA deficiency did not impact the in vitro differentiation of iT_R cells nor did it affect the fraction of recent thymic immigrants amongst peripheral T_{conv} and T_R cells. Instead, it was associated with increased apoptosis of T_R and T_{conv} cells, with increased turnover of the latter but not the former cells. A disproportionately constrained T_R 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 T_R cell T cell receptor repertoire³⁷.

T_R cell depletion in LRBA deficiency is compounded by impaired T_R cell phenotype and function, with decreased expression of key effector proteins involved in T_R cell suppression such as CD25 and CTLA-4³⁸. LRBA localizes to cytosol at lysosomes, the trans-Golgi network, the ER, the perinuclear ER, and endocytic vacuoles³⁹. It is thus spatially segregated from FOXP3, whose expression is exclusively nuclear⁷. In searching for a

mechanism that accounts for adverse impact of LRBA deficiency on T_R cells, the finding of impaired activation of mTOR complexes invoked deranged metabolic sensing in disease pathogenesis⁴⁰. In particular, defective mTORC1 cell activation may provide one mechanism by which LRBA deficiency impairs T_R cell function. In mice, T_R 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 autoimmunity³³. mTORC1 directs T_R cell lipid metabolism, relevant to their energy generation and suppressor function³³. It impacts the expression of several T_R cell markers, most notably CTLA-4, which may contribute to immune dysregulation upon mTORC1 deficiency³³.

Mechanisms by which LRBA deficiency impairs mTOR activation may involve defective autophagy, as originally described by Lopez-Herrera et al¹⁷. Autophagy regulates energy metabolism in T cells, and its dysfunction may result in decreased activation of mTOR complexes and attenuated phosphorylation of downstream targets including S6 and 4E-BP1^{41, 42}. Alternatively, LRBA may act as a scaffold that enables the assembly and activation of mTOR complexes and/or recruitment of downstream intermediates such as AKT, as has been described for other scaffold proteins⁴³.

LRBA deficiency is associated with intense autoantibody responses even in the context of B cell lymphopenia and hypogammaglobulinemia. Our studies have identified increase T_{FH} and decreased T_{FR} cell frequencies in the peripheral blood of LRBA deficient subjects, consistent with ineffective regulation of antibody responses. T_{FR} cell deficiency and its associated T_{FH} cell dysregulation has been implicated in the development of autoantibodies in immune dysregulatory diseases, such as IPEX^{25–29}. We hypothesize that this abnormality is a key mechanism in the development of humoral autoimmunity in LRBA deficiency. While the precise cause of T_{FH} dysregulation remains to be mapped, enhanced FOXO1/3a induced activation of BCL6 due to decreased negative regulation of the former by pAKTS473 may provide one putative mechanism⁴⁴. Further studies would be required to validate such a link.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR autosomal recessive

CVID	Common variable immunodeficiency
dsDNA	double stranded DNA
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescence-activated cell sorting
FOXP3	forkhead box P3
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mTORC1 and mTORC2	mammalian target of rapamycin 1 and 2 complexes
PBMC	peripheral blood mononuclear cell
RTE	recent thymic emigrants
T_{eff}	T effector
T_R	T regulatory
SLE	systemic lupus erythematosus
WES	Whole exome sequencing

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Key messages

- LRBA deficiency results in profound T regulatory (T_R) cell deficiency and impaired phenotype and function.
- LRBA deficiency may present with Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked-like disease, reflecting their profound T_R cell deficiency.
- LRBA deficient subjects exhibit high frequency of memory T cells and a dysregulated T follicular helper cell compartment with increased autoantibody production.

Figure 1.

A. Pedigrees of the LRBA-deficient families. Generations are designated by Roman numerals (I–II) and individuals by Arabic numerals. Double lines connecting parents indicate consanguinity. Proband in the respective families are indicated by P1–P6 labeling. Squares, males; circles, females; filled symbols, patients. **B.** Schematic representation of LRBA protein showing the respective domains and the location of patient mutations. **C.** Sanger sequencing chromatograms showing genomic DNA mutations of patient P1 and P2 as compared to the equivalent DNA sequences of healthy controls (Ctrl). **D.** Western blot analysis of LRBA protein expression in fibroblasts of patient P1 and lymphocytes of patients P2, P3, P5, and P6 as compared to those of controls. The blots were reprobbed for β-actin as a positive control.

Figure 2. LRBA deficiency leads to defect in T_R cell frequency and phenotype

A. Representative dot plot analysis of FOXP3 expression in patient P1 vs. a control subject.

B. Percent CD4⁺FOXP3⁺ T cells in the peripheral blood of healthy controls (n=8; open

circles) and LRBA deficient patients (n=6; closed circles). **C.** Representative flow cytometric histogram plots of FOXP3, CD25, CTLA4 and Helios expression in FOXP3⁺CD4⁺ T cells from controls (dotted line) and LRBA deficient patients (solid line). Gray area represents control isotype staining. **D.** Mean fluorescence intensity of the

respective T_R cell marker in patient and control subjects. ** p<0.01, *** p<0.001 by unpaired two-tailed Student's *t*-test.

Figure 3. LRBA-deficient T_R cells have decreased in vitro suppressive ability

A. Flow cytometric analysis of CellTrace Violet proliferation dye dilution in T_{eff} ($CD4^+CD25^-$) cells stimulated with anti-CD2/CD3/CD28 mAb-coated bead in the absence or presence of T_R cells ($CD4^+CD25^+CD127^{low}$) from healthy controls or LRBA deficient patients. **B.** Cumulative Results of *in vitro* suppression assays in patient and control subjects. N=6 individuals per group, **** $p < 0.0001$ by unpaired two-tailed Student's *t*-test. **C.** IL-10 production in patient and control T_R cells at baseline and following stimulation with the α CD2/CD3/CD28 or PMA + Ionomycin (Iono) for 4 days. N.D.: Not detected. * $P < 0.05$ by 2-way ANOVA with post-test analysis.

Figure 4. Increased frequency of memory T cells in LRBA-deficient subjects

A. Flow cytometric analysis of circulating CD45RO⁻CD45RA⁺ and CD45RO⁺CD45RA⁻ CD4⁺ T cells in an LRBA-deficient and a control subject. **B.** Frequency of activated CD45RO⁺CD45RA⁻ within the peripheral blood CD4⁺ T cell pool of LRBA-deficient and control subjects. **C.** Flow cytometric analysis of circulating CD45RO⁻CD45RA⁺ and CD45RO⁺CD45RA⁻ CD8⁺ T cells in an LRBA-deficient and a control subject. **D.** Frequency of activated CD45RO⁺CD45RA⁻ within the peripheral blood CD8⁺ T cell pool of LRBA-deficient and control subjects. **E.** Flow cytometric analysis of circulating TEMRA CD45RA⁺CCR7⁻ and naïve CD45RA⁺CCR7⁺ CD8⁺ T cells in an LRBA-deficient and a control subject. **F.** Frequency of circulating TEMRA CD45RA⁺CCR7⁻ and naïve CD45RA⁺CCR7⁺ within the peripheral blood CD8⁺ T cell pool of LRBA-deficient and control subjects. *p<0.05, ***p<0.001 by Student's unpaired two tailed *t* test.

Figure 5. Increased autoantibodies and dysregulated T_{FH} cell response in LRBA-deficient patients

A. Heat map display of autoantibody reactivity against self-antigens in sera of LRBA-deficient and control subjects, a patient with IPEX and a patient with SLE. A value of 1 (black) is equal to the control average + 1 standard deviation (SD). **B.** Flow cytometric analysis of circulating T_{FH} (CD4⁺CXCR5⁺PD-1^{hi}) cells in patient and control subjects. **C.** Frequency of T_{FH} cells within the peripheral blood CD4⁺ T cells of LRBA-deficient and control subjects. **D.** Flow cytometric overlays of the T_{FH} markers BCL6, ICOS and CD40L within the peripheral blood CD4⁺CXCR5⁺PD-1⁺ cell pool of LRBA-deficient and control subjects. **E.** Flow cytometric analysis of circulating T_{FR} cells. T cells within the CD4⁺CXCR5⁺PD-1^{hi} gate were analyzed for Foxp3 expression. **F.** Frequencies of T_{FR} cells within the peripheral blood CD4⁺CXCR5⁺PD-1⁺ cell pool. *p<0.05, ***p<0.001 by Student's unpaired two tailed *t* test.

Figure 6. LRBA deficiency does not affect the *in vitro* generation of FOXP3⁺ iT_R cells

A. Representative flow cytometric analyses of CD45RA/RO markers in purified CD4⁺ T cells (upper panel) and in cell sorted CD3⁺CD4⁺CD25⁻CD45RO⁻CD45RA⁺ T cells (lower panels) of a control and an LRBA-deficient subject. **B.** Representative flow cytometric analyses of Foxp3 expression in T cells sorted as in panel A and stimulated in culture with anti-CD2/3/28 mAbs in the absence or presence of TGF- β 1. **C.** Collated frequencies of FOXP3⁺ iT_R cells in control and LRBA-deficient subjects (N=4–5/group). Results are representative of 2 independent experiments.

Figure 7. Increased turnover of LRBA-deficient T cells

A, C E. Flow cytometric analysis of AnnexinV (**A**), Ki67 (**B**) and CD31 (**C**) staining in ex-vivo CD4⁺ T_{conv} and T_R cells from LRBA-deficient and control subjects. **B, D, F.** Frequencies of AnnexinV⁺ (**B**), Ki67⁺ (**D**) and CD31⁺ (**F**) cells within T_{conv} and T_R cell populations of LRBA-deficient and control subjects. N=4–5 patients and 5–8 controls/group. *p<0.05, **p<0.01 by 2-way ANOVA with post-test analysis.

Figure 8. Impaired mTORC1 and mTORC2 activities in LRBA-deficient T_{conv} and T_R cells
A. Flow cytometric analysis of pS6 of conventional (T_{conv}) and regulatory (T_R) T cells from LRBA-deficient and control subjects before after anti-CD2/3/28 stimulation. **B.** Mean Fluorescence Intensity (MFI) of pS6 within T_{conv} and T_R cells of LRBA-deficient and control subjects before and after anti-CD2/3/28 stimulation. **C.** Flow cytometric analysis of p4E-BP1 of T_{conv} and T_R cells from LRBA-deficient and control subjects after anti-CD2/3/28 stimulation. **D.** MFI of p4E-BP1 within T_{conv} and T_R cells of LRBA-deficient and control subjects before and after anti-CD2/3/28 stimulation. **E.** Flow cytometric analysis of p-AKT (S473) of T_{conv} and T_R cells from LRBA-deficient and control subjects after anti-CD2/3/28 stimulation. **F.** MFI of p-AKT (S473) within T_{conv} and T_R cells of LRBA-deficient and control subjects before and after anti-CD2/3/28 stimulation. *p<0.05, **p<0.01 by 2-way ANOVA with post-test analysis.