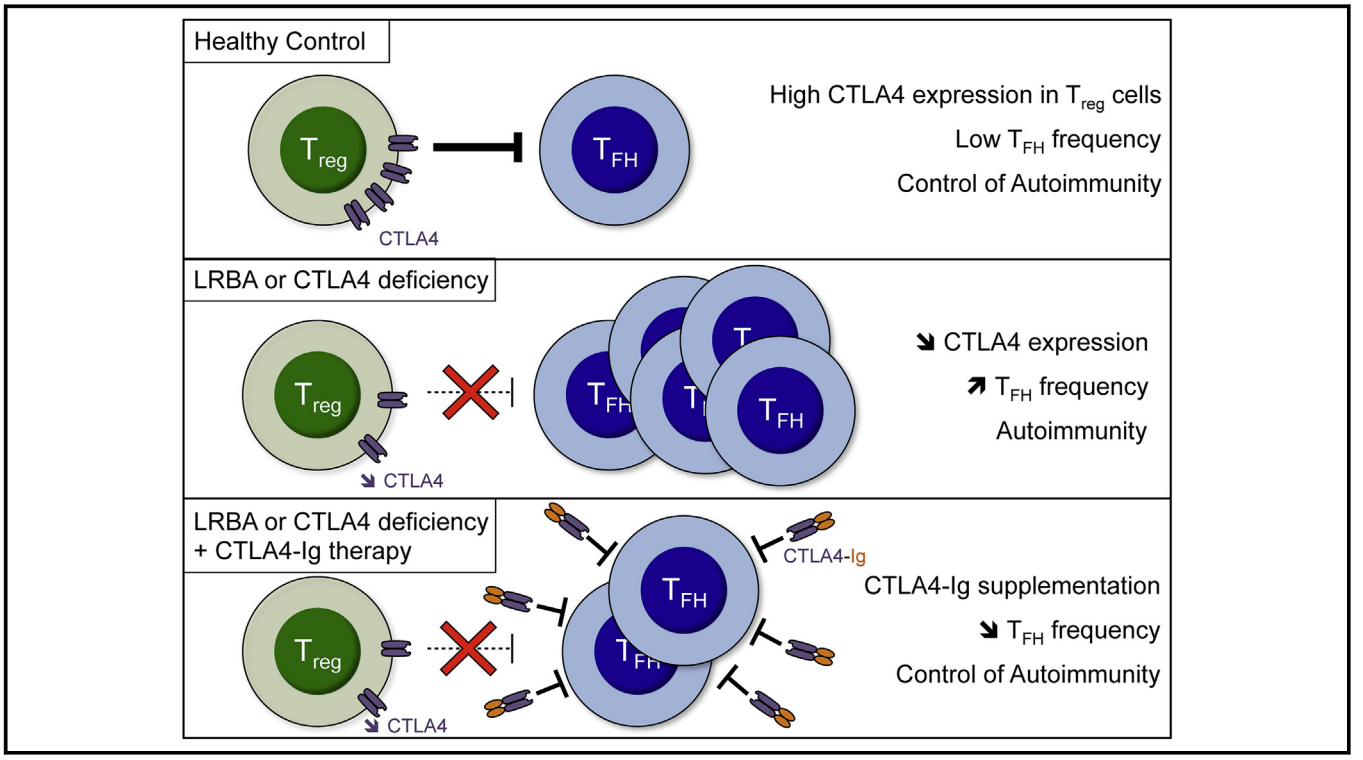
Exaggerated follicular helper T-cell responses in patients with LRBA deficiency caused by failure of CTLA4-mediated regulation

Fayhan J. Alroqi, MD,[a]* Louis-Marie Charbonnier, PhD,[a]* Safa Baris, MD,[b] Ayca Kiykim, MD,[b] Janet Chou, MD,[a] Craig D. Platt, MD, PhD,[a] Abdulrahman Algassim, MD,[a] Sevgi Keles, MD,[a,c] Bandar K. Al Saud, MD,[d] Fowzan S. Alkuraya, MD,[e] Michael Jordan, MD,[f,g] Raif S. Geha, MD,[a] and Talal A. Chatila, MD, MSc[a]

Boston, Mass, Istanbul and Konya, Turkey, Riyadh, Saudi Arabia, and Cincinnati, Ohio

GRAPHICAL ABSTRACT



Background: LPS-responsive beige-like anchor protein (LRBA) and cytotoxic T lymphocyte–associated antigen 4 (CTLA4) deficiencies give rise to overlapping phenotypes of immune dysregulation and autoimmunity, with dramatically increased frequencies of circulating follicular helper T (cT_{FH}) cells.

Objective: We sought to determine the mechanisms of cT_{FH} cell dysregulation in patients with LRBA deficiency and the utility of

From [a]the Division of Immunology, Boston Children's Hospital, and the Department of Pediatrics, Harvard Medical School, Boston; [b]the Division of Pediatric Allergy/Immunology, Marmara University, Istanbul; [c]the Division of Pediatric Allergy and Immunology, Meram Medical Faculty, Necmettin Erbakan University, Konya; the Departments of [d]Pediatrics and [e]Genetics, King Faisal Specialist Hospital and Research Center, Riyadh; and the Divisions of [f]Bone Marrow Transplantation and Immune Deficiency and [g]Immunobiology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati.

*These authors contributed equally to this work.

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Corresponding author: Talal A. Chatila, MD, MSc, Division of Immunology, Boston Children's Hospital, One Blackfan Circle, Boston, MA 02115. E-mail: talal.chatila@childrens.harvard.edu.

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monitoring cT_{FH} cells as a correlate of clinical response to CTLA4-Ig therapy.

Methods: cT_{FH} cells and other lymphocyte subpopulations were characterized. Functional analyses included *in vitro* follicular helper T (T_{FH}) cell differentiation and cT_{FH}/naive B-cell cocultures. Serum soluble IL-2 receptor α chain levels and *in vitro* immunoglobulin production by cultured B cells were quantified by using ELISA.

Results: cT_{FH} cell frequencies in patients with LRBA or CTLA4 deficiency sharply decreased with CTLA4-Ig therapy in parallel with other markers of immune dysregulation, including soluble IL-2 receptor α chain, $CD45RO^+CD4^+$ effector T cells, and autoantibodies, and this was predictive of favorable clinical responses. cT_{FH} cells in patients with LRBA deficiency were biased toward a T_H1-like cell phenotype, which was partially reversed by CTLA4-Ig therapy. LRBA-sufficient but not LRBA-deficient regulatory T cells suppressed *in vitro* T_{FH} cell differentiation in a CTLA4-dependent manner. LRBA-deficient T_{FH} cells supported *in vitro* antibody production by naive LRBA-sufficient B cells.

Conclusions: cT_{FH} cell dysregulation in patients with LRBA deficiency reflects impaired control of T_{FH} cell differentiation because of profoundly decreased CTLA4 expression on regulatory T cells and probably contributes to autoimmunity in patients with this disease. Serial monitoring of cT_{FH} cell frequencies is highly useful in gauging the clinical response of LRBA-deficient patients to CTLA4-Ig therapy. (J Allergy Clin Immunol 2018;141:1050-9.)

Key words: *Autoantibodies, LPS-responsive beige-like anchor, cytotoxic T lymphocyte–associated antigen 4, regulatory T cells, follicular helper T cells, follicular regulatory T cells*

Abbreviations used

cT_{FH}:	Circulating follicular helper T
CTLA4:	Cytotoxic T lymphocyte–associated antigen 4
FOXP3:	Forkhead box P3
ICOS:	Inducible T-cell costimulator
IPEX:	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
iT_{FH}:	Induced follicular helper T
IVIG:	Intravenous immunoglobulin
LRBA:	LPS-responsive beige-like anchor
PD-1:	Programmed cell death protein 1
sCD25:	Soluble CD25
SLE:	Systemic lupus erythematosus
T_{FH}:	Follicular helper T
T_{FR}:	Follicular regulatory T
Treg:	Regulatory T
WES:	Whole-exome sequencing

Follicular helper T (T_{FH}) cells are a distinct subset of T cells with a crucial role in humoral adaptive immunity.[1] This specific T_H lineage has a unique phenotype, expressing high levels of CXCR5, programmed cell death protein 1 (PD-1), inducible T-cell costimulator (ICOS), and CD40 ligand.[2,3] T_{FH} cells constitutively express high levels of B-cell lymphoma 6, whereas other T_H cell populations, including T_H1, T_H2, and T_H17 cells, express high levels of the antagonizing transcription factor B lymphocyte-induced maturation protein-1 (Blimp-1).[4]

Various signaling pathways have been implicated in T_{FH} cell differentiation, including signals from dendritic cells, B cells, cytokines (IL-6 and IL-21 in mice and IL-12, IL-23, and TGF-β in human subjects) and surface molecules (ICOS, CD28, CD40 ligand, PD-1, B- and T-lymphocyte attenuator [BTLA], and SLAM-associated protein [SAP]).[1] T_{FH} cells are essential for germinal center formation and B-cell differentiation into long-lived memory B cells and plasma cells within secondary lymphoid tissues. Upregulation of CXCR5 and downregulation of CCR7 guide T_{FH} cell migration into the B-cell follicle.[1] The interaction between T_{FH} cells and their cognate B cells provides fundamental signals for high-affinity antibodies production through affinity maturation and class-switch recombination. The importance of the T_{FH} cell lineage in promoting antibody responses was established by the observation of defective antibodies class-switching in mice lacking T_{FH} cells,[5] whereas unbridled T_{FH} cell responses trigger humoral autoimmunity caused by generation of autoantibodies by autoreactive B cells.[5-10]

Circulating follicular helper T (cT_{FH}) cells were recently identified in human subjects as reflective of T_{FH} cells.[3,11] Increased numbers of cT_{FH} cells have been reported in patients with several human autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren syndrome, and autoimmune thyroid disease.[12-16] Reciprocally, decreased numbers of cT_{FH} cells have been found in patients with several monogenic immunodeficiency disorders associated with humoral deficiency.[17]

Follicular regulatory T (T_{FR}) cells, which originate from forkhead box P3 $(FOXP3)^+$ T cells and express high levels of CXCR5, play a pivotal role in controlling immune dysregulation by regulating T_{FH} cell and activated B-cell responses through the inhibitory effect of cytotoxic T lymphocyte–associated antigen 4 (CTLA4).[18-22] CTLA4 deletion in mice results in an exaggerated B-cell response and increased T_{FH} cells frequencies.[21-24] A number of inherited disorders of immune dysregulation that potentially affect T_{FR} cell function result in high cT_{FH} cell frequencies, which positively correlate with autoantibody production. The best characterized of these are monogenic defects involving the LPS-responsive beige-like anchor (LRBA)–CTLA4 pathway.[25-30] LRBA deficiency is a primary immunodeficiency characterized by recurrent infections with hypogammaglobulinemia, giving rise to a common variable immunodeficiency–like phenotype and immunodysregulation with autoimmunity, including inflammatory bowel disease, autoimmune endocrinopathies, and cytopenias.[25,26,29-31] LRBA regulates the intracellular trafficking of CTLA4.[30] Its deficiency results in dysregulation of the T_{FH} cell response and is associated with intense immune dysregulation and autoantibody production.[29] The precise mechanism for T_{FH} cell dysregulation in patients with LRBA deficiency, whether it involves enhanced T_{FH} cell differentiation, impaired T_{FR} cell regulation, or both, remains unclear. CTLA4-Ig therapy has emerged as an effective treatment for the immune dysregulation associated with both gene defects.[30] The capacity to rapidly and sensitively monitor the immune status of these patients and their response to CTLA4-Ig therapy would be of great clinical utility in optimizing their care.

In this report we examined the mechanisms of T_{FH} cell dysregulation in patients with LRBA deficiency and the utility of using cT_{FH} cell frequencies as a clinical indicator of response to treatment with CTLA4-Ig. Serial cT_{FH} cell analysis was

carried out on LRBA-deficient patients before and after starting CTLA4-Ig therapy. Our results show that T_{FH} cell dysregulation in patients with LRBA deficiency was not due to an intrinsic abnormality in T_{FH} cells. Rather, it reflects failure of LRBA-deficient regulatory T (Treg) cells to control T_{FH} cell differentiation. cT_{FH} cells were found to be a sensitive marker of the clinical response to CTLA4-Ig therapy. Our finding supports the use of cT_{FH} cell frequencies as a marker for monitoring the clinical status of LRBA-deficient patients and their response to therapy.

METHODS

Patients

Patients P1 and P2 are 2 previously described Saudi Arabian siblings with LRBA deficiency caused by a homozygous deletion in the BEACH domain of *LRBA* that abolished protein expression (patients P5 and P6; family C in our original report).[29]

Patient P3 is an 11-year-old girl with chronic immune dysregulation who received a diagnosis of LRBA deficiency because of exon 57 deletion, as confirmed by means of genomic analysis, cDNA sequencing, and absent LRBA protein on flow cytometry and immunoblotting (see Fig E1 in this article's Online Repository at www.jacionline.org). Her clinical presentation is detailed in the Methods section in this article's Online Repository at www.jacionline.org.

Patient P4 is a 6-year-old Saudi Arabian boy who had severe Crohn-like illness and type 1 diabetes and was found on whole-exome sequencing (WES) to have a 4-bp deletion in *LRBA* (c.4757_4760del, p.L1586fs). LRBA deficiency was confirmed by absent protein expression on flow cytometry (see Fig E2 in this article's Online Repository at www.jacionline.org).

Patients P5 and P6 are Turkish brothers with newly diagnosed LRBA deficiency caused by a 1-bp deletion in exon 54 of *LRBA*, as confirmed by means of genomic analysis, leading to a frame shift and premature stop codon (c.7885delA, p.R2629fs). LRBA deficiency was confirmed by near-absent protein expression on flow cytometry (see Fig E3 in this article's Online Repository at www.jacionline.org).

Patients P7 to P9 were found to have heterozygous mutations in *CTLA4* (see Fig E4 in this article's Online Repository at www.jacionline.org). Their clinical presentations are detailed in the Methods section in this article's Online Repository. A patient with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome caused by an A384T amino acid substitution in FOXP3 was identified by means of exome analysis, followed by Sanger sequencing of the mutation site in the *FOXP3* gene. A subject with a diagnosis of SLE was identified at the Rheumatology clinic at Boston Children's Hospital. Control subjects were age group matched.

All study participants were recruited by using written informed consent approved by 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 used is provided in the Methods section in this article's Online Repository. Whole blood was incubated with mAbs against surface markers for 30 minutes on ice. Intracellular staining with FOXP3 and CTLA4 was performed by using eBioscience Fixation/Permeabilization (eBioscience, San Diego, Calif), according to the manufacturer's instructions. Indirect intracellular staining for LRBA was performed on freshly isolated PBMCs by using BD Biosciences Fixation/Permeabilization buffer (BD Biosciences, San Jose, Calif) with polyclonal rabbit anti-LRBA antibodies (Sigma-Aldrich, St Louis, Mo) or Rabbit IgG XP (R) isotype control (Cell Signaling, Danvers, Mass), followed by secondary detection with Brilliant Violet 421 Donkey anti-Rabbit IgG (BioLegend, San Diego). For chemokine receptor staining in cT_{FH} cells, PBMCs were isolated by using Ficoll-Paque Plus gradient, and surface staining for PD-1, CXCR5, CXCR3, and CCR6 was performed for 30 minutes on ice. For cytokine detection among T_{FH} cells, PBMCs were stimulated *in vitro* for 4 hours in complete RPMI medium with Golgi plug (1:1000), phorbol 12-myristate 13-acetate (50 ng/mL), and ionomycin (500 ng/mL). Cells were washed and incubated with mAbs against surface markers for 30 minutes on ice and permeabilized with BD Biosciences Fixation/Permeabilization buffer, and intracellular staining with mAbs against cytokines was performed. For characterization of circulating B-cell subsets, previously frozen PBMCs were stained for the surface antigens CD19, CD27, and IgD. Data were collected with a Fortessa cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore).

WES

WES was performed on genomic DNA of the probands P3, P4, P5, and P9, as described in earlier reports.[25,29]

Sanger sequencing analysis

LRBA and *CTLA4* sequences were amplified from genomic DNA and cDNA by using PCR and sequenced bidirectionally by using dye terminator chemistry.[29] Primers used in amplification reactions are available on request.

Immunoblotting

Protein lysates derived from lymphocytes were resolved on SDS-PAGE gels and transferred to nitrocellulose filters, as described. Immunoblots were carried out with polyclonal rabbit anti-LRBA antibody (Sigma-Aldrich). The blots were reprobed with polyclonal rabbit anti-dedicator of cytokinesis 8 (DOCK8) antibody (Sigma-Aldrich).

T_{FH} cell and naive B-cell coculture

cT_{FH} and naive B cells were isolated from the peripheral blood of patient P3 and her fully HLA-matched healthy sister by using fluorescence-activated cell sorting. The cells were mixed as indicated and cultured for 12 days in the presence of endotoxin-reduced staphylococcal enterotoxin B at 1 μg/mL in RPMI 1640 complete medium supplemented with 10% heat-inactivated FBS.[32] At the end of the incubation period, IgM and IgG concentrations in culture supernatants were measured by using ELISA.

In vitro generation of induced T_{FH} cells

$CD4^+$ T cells were isolated from PBMCs by means of negative selection with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Naive $CD3^+CD4^+CCR7^+CD45RA^+$ cells were purified from $CD4^+$ T cells by means of cell sorting on a FACSAria (purity > 98%). Naive CD4 T cells were seeded at a concentration of 5×10^4 per well of a 96-well plate and stimulated with recombinant human IL-12, IL-23, and TGF-β1 in the presence or absence of Treg cells, CTLA4-Ig, and anti-CTLA4-mAb (Biolegend), as indicated.[33] The cells were cultured for 4 days, at the end of which they were stained for T_{FH} cell markers.

Autoantibody assays

For autoantibody detection, plasma aliquots from patients and control subjects were analyzed by using microarrays spotted with 84 autoantigens (University of Texas Southwestern Medical Center, Genomic and Microarray Core Facility, Dallas, Tex), as previously described.[34] Data were normalized to those of healthy control subjects.

Statistical analysis

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.

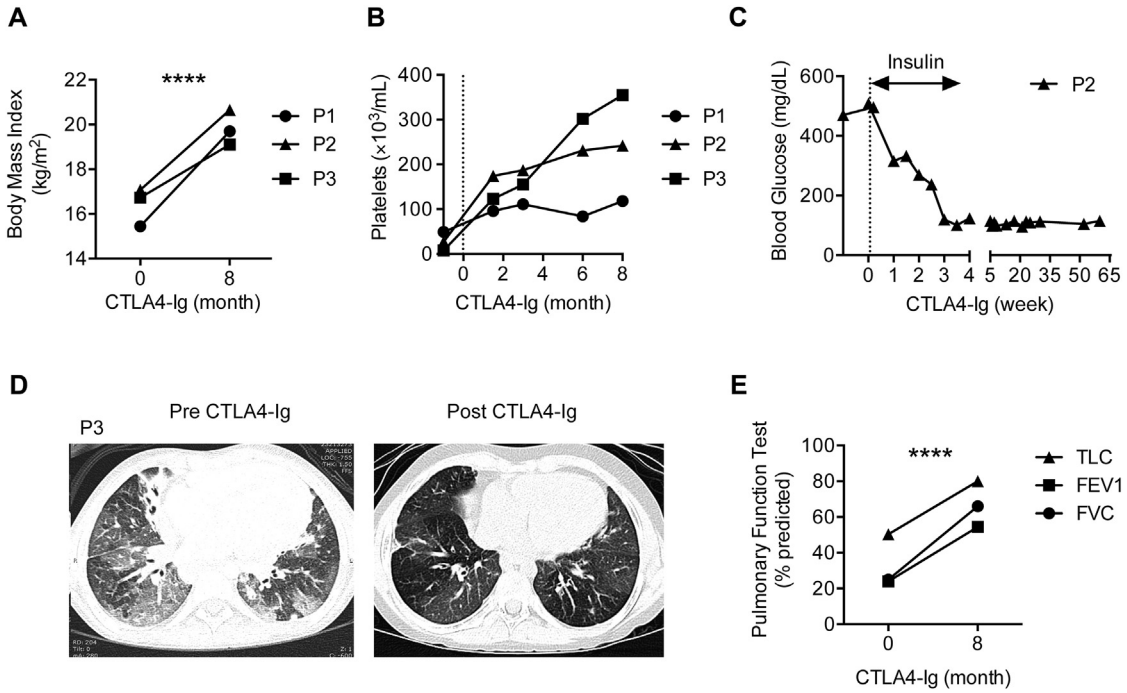

FIG 1. Clinical response of LRBA-deficient patients to CTLA4-Ig therapy. **A** and **B,** Body mass index (Fig 1, *A*) and platelet counts (Fig 1, *B*) of LRBA-deficient patients before and after treatment with CTLA4-Ig. **C,** Serum glucose levels and insulin use in patient P2: status after CTLA4-Ig therapy. **D** and **E,** High-resolution chest computed tomographic scans (Fig 1, *D*) and pulmonary function tests (Fig 1, *E*) of patient P3 before and after treatment with CTLA4-Ig. *FVC,* Forced vital capacity; *TLC,* total lung capacity. ****$P < .0001$, 2-way ANOVA with posttest analysis.

RESULTS

Three patients with definitive LRBA deficiency were studied for their response to CTLA4 therapy. Details of the clinical and immunologic findings of the 3 patients are shown in Tables E1 and E2 in this article's Online Repository at www.jacionline.org, respectively. The 3 patients exhibited severe immune dysregulation with autoimmune cytopenias, chronic end-organ inflammation, and damage, especially affecting the lungs (patients P1 and P3) and the gut (patients P1 and P2). All patients had marked clinical response to CTLA4-Ig therapy with decreased disease symptomatology, weight gain, and resolution of their thrombocytopenia (Fig 1, *A* and *B*). Patients P1 and P2, who had colitis, showed good clinical response with decreased diarrhea. Patient P2, who had type 1 diabetes shortly before initiation of therapy, had resolution of her insulin dependence and normalization of her blood glucose (Fig 1, *C*). Patient P3, who had severe lung disease and was oxygen dependent, responded very favorably to CTLA4-Ig therapy with marked improvement in her lung imaging and function and resolution of her oxygen dependency (Fig 1, *D* and *E*).

Previously, we documented that patients with LRBA deficiency exhibit dysregulated cT_{FH} cells,[29] a phenotype related to the profound deficiency of CTLA4 expression by LRBA-deficient Treg cells.[21,22,29] Flow cytometric analysis of peripheral blood T lymphocytes from the 3 patients demonstrated a markedly increased number of $CD4^+FOXP3^-PD1^+CXCR5^+$ cT_{FH} cells (Fig 2, *A*). The frequency of cT_{FH} cell and CTLA4 expression of Treg cells of 3 other patients with LRBA deficiency (patients P4-P6) were also analyzed. They also exhibited a high frequency of cT_{FH} cells in association with low CTLA4 expression on their Treg cells (see Figs E2 and E3). Importantly, the frequency of cT_{FH} cells significantly decreased after CTLA4-Ig therapy (Fig 2, *A* and *B*). CTLA4-Ig therapy also resulted in a decrease in other markers of inflammation, including serum soluble CD25 (sCD25; Fig 2, *C*). The frequency of circulating naive $CD4^+$ T cells ($CD4^+CD45RA^+CCR7^+$) increased after CTLA4-Ig therapy, which is indicative of more effective immunoregulation (Fig 2, *D*). The decrease in cT_{FH} cell numbers on CTLA4-Ig therapy correlated well with that of sCD25, validating the monitoring of these cells as a proxy for the immune dysregulatory status of the patients (Fig 2, *E*). In addition to their increased frequency, cT_{FH} cells of LRBA-deficient subjects also had increased expression of the cT_{FH} cell markers ICOS and PD-1 compared with those of control subjects, which is consistent with them being activated.[11,35] The expression levels of both markers were normalized after therapy with CTLA4-Ig (Fig 2, *F* and *G*).

To further characterize the phenotype of cT_{FH} cells in LRBA-deficient patients and also the effect of CTLA4-Ig treatment, we evaluated the expression of chemokine receptors and the capacity of cT_{FH} cells to secrete cytokines. cT_{FH} cells of LRBA-deficient patients expressed high levels of the T_H1-associated chemokine receptor CXCR3 and IFN-γ, markers that define T_H1-biased T_{FH} ($T_{FH}1$) cells.[11,35] In contrast, expression of the T_H17-associated chemokine receptor CCR6 and IL-17, markers that define T_H17-biased T_{FH} ($T_{FH}17$) cells,[11,35] was markedly decreased in cT_{FH} cells from patients compared with those from control subjects (Fig 3). Some of these abnormalities, such as increased CXCR3 expression, persisted despite CTLA4-Ig therapy and probably reflected either attributes intrinsic to LRBA-deficient T_{FH} cells or ongoing T_H1 cell inflammation in the

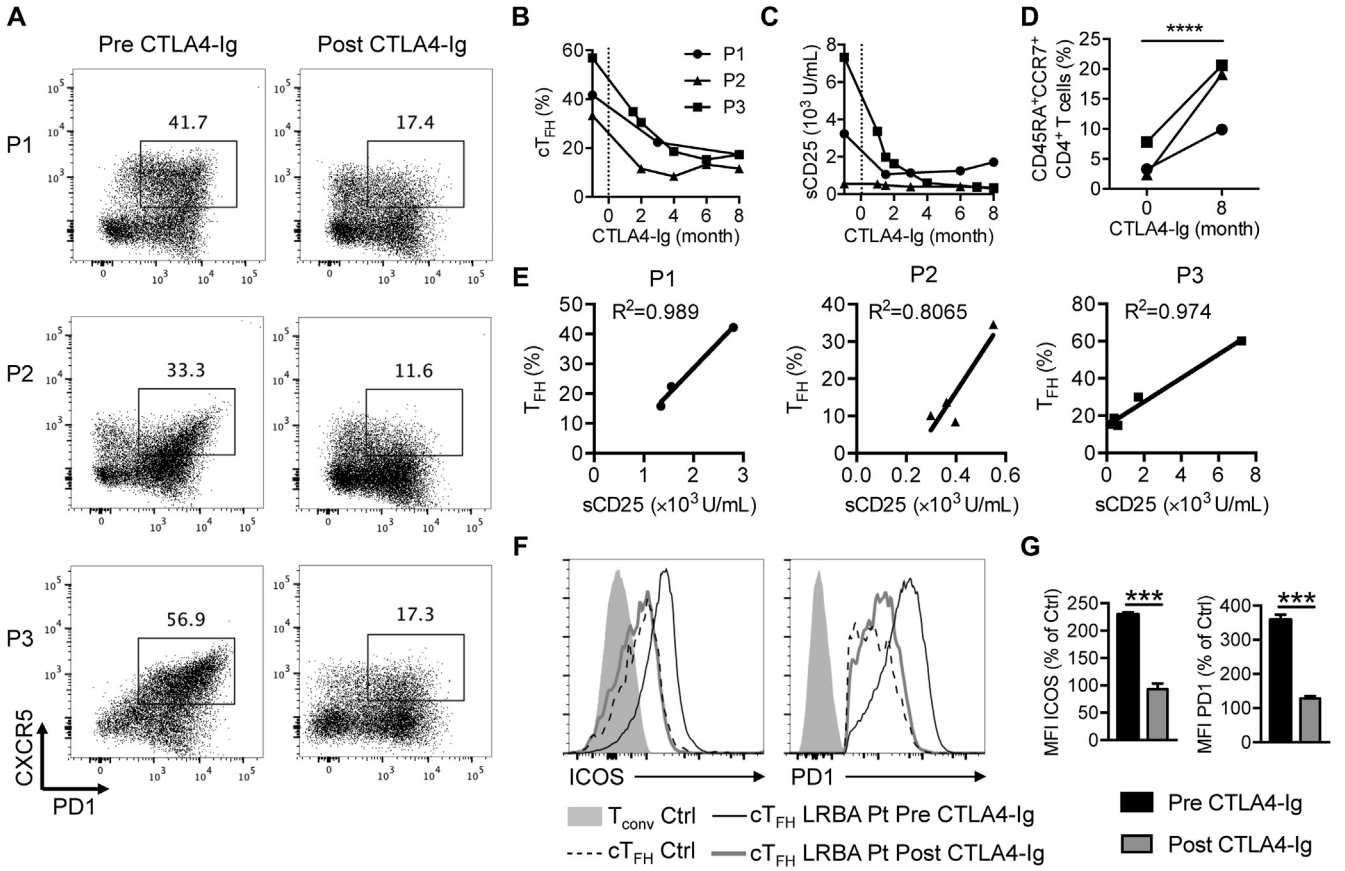

FIG 2. T_{FH} cell frequency in LRBA-deficient patients correlates with other markers of immune dysregulation. **A,** Flow cytometric analyses of CXCR5 and PD-1 expression in $CD4^+$ T cells in LRBA-deficient patients before and after treatment with CTLA4-Ig. **B-D,** cT_{FH} cell frequencies (Fig 2, *B*), serum sCD25 levels (Fig 2, *C*), and naive $CD4^+CD45RA^+CCR7^+$ T-cell frequencies (Fig 2, *D*) in LRBA-deficient patients before and after treatment with CTLA4-Ig. **E,** Correlation between cT_{FH} cell frequencies and sCD25 levels in LRBA-deficient patients. **F,** Flow cytometric analysis of ICOS and PD-1 expression on control cT_{FH} cells and on patients' cT_{FH} cells before and after CTLA4-Ig therapy. **G,** Relative mean fluorescence intensity *(MFI)* of ICOS and PD-1 expression on cT_{FH} cells from patients versus control subjects. ***$P < .001$ and ****$P < .0001$, Student 2-tailed *t* test.

host. In contrast, expression of IL-21 and IL-4 was similar in cT_{FH} cells from patients and control subjects and appeared unaffected by CTLA4-Ig therapy.

We have previously reported that patients with LRBA deficiency have profound Treg cell abnormalities, including decreased Treg cell frequencies and reduced expression of several Treg cell markers, including CTLA4.[29] In view of this immune dysregulation, we analyzed the correlation of the magnitude of CTLA4 expression on Treg cells and the frequency of cT_{FH} cells. To that end, we examined the frequency of cT_{FH} cells in 3 patients with heterozygous loss-of-function mutations in *CTLA4*. Their mutational analysis is shown in Fig E4, and their clinical and immunologic findings are detailed in Tables E1 and E3 in this article's Online Repository at www.jacionline.org, respectively. Consistent with the role of CTLA4 deficiency in dysregulated cT_{FH} cells in patients with LRBA deficiency, patients with heterozygous loss-of-function mutations in *CTLA4* also manifested increased cT_{FH} cell frequencies (Fig 4, *A* and *B*). Overall, cT_{FH} cell frequencies were inversely correlated with the CTLA4 mean fluorescence intensity on Treg cells of LRBA- and CTLA4-deficient subjects, reflecting the role of CTLA4 in controlling T_{FH} cell differentiation. Subjects with complete LRBA deficiency associated with near-complete absence of CTLA4 expression on Treg cells manifested the highest cT_{FH} cell frequencies, whereas those with heterozygous loss-of-function *CTLA4* mutations had a more moderate increase in cT_{FH} cell frequencies (Fig 4, *C*). Similar to the case of CTLA4-Ig−treated LRBA-deficient subjects, treatment of a patient with CTLA4 deficiency with CTLA4-Ig resulted in a decrease in cT_{FH} cell numbers, which is consistent with the role of CTLA4 deficiency in cT_{FH} cell dysregulation in patients with both disorders (Fig 4, *D* and *E*).

To determine whether LRBA deficiency impaired T_{FH} cell differentiation, we tested the capacity of LRBA-sufficient and LRBA-deficient naive $CD4^+$ T cells to differentiate into induced follicular helper T (iT_{FH}) cells on stimulation with IL-12, IL-23, and TGF-β1 in the presence or absence of CTLA4-Ig.[33] $CD4^+$ T cells from patients and control subjects were enriched by means of magnetic bead separation, and naive $CD4^+$ T cells were purified to greater than 99% purity by means of cell sorting (see Fig E5 in this article's Online Repository at www.jacionline.org). LRBA-sufficient and LRBA-deficient T cells were found to be

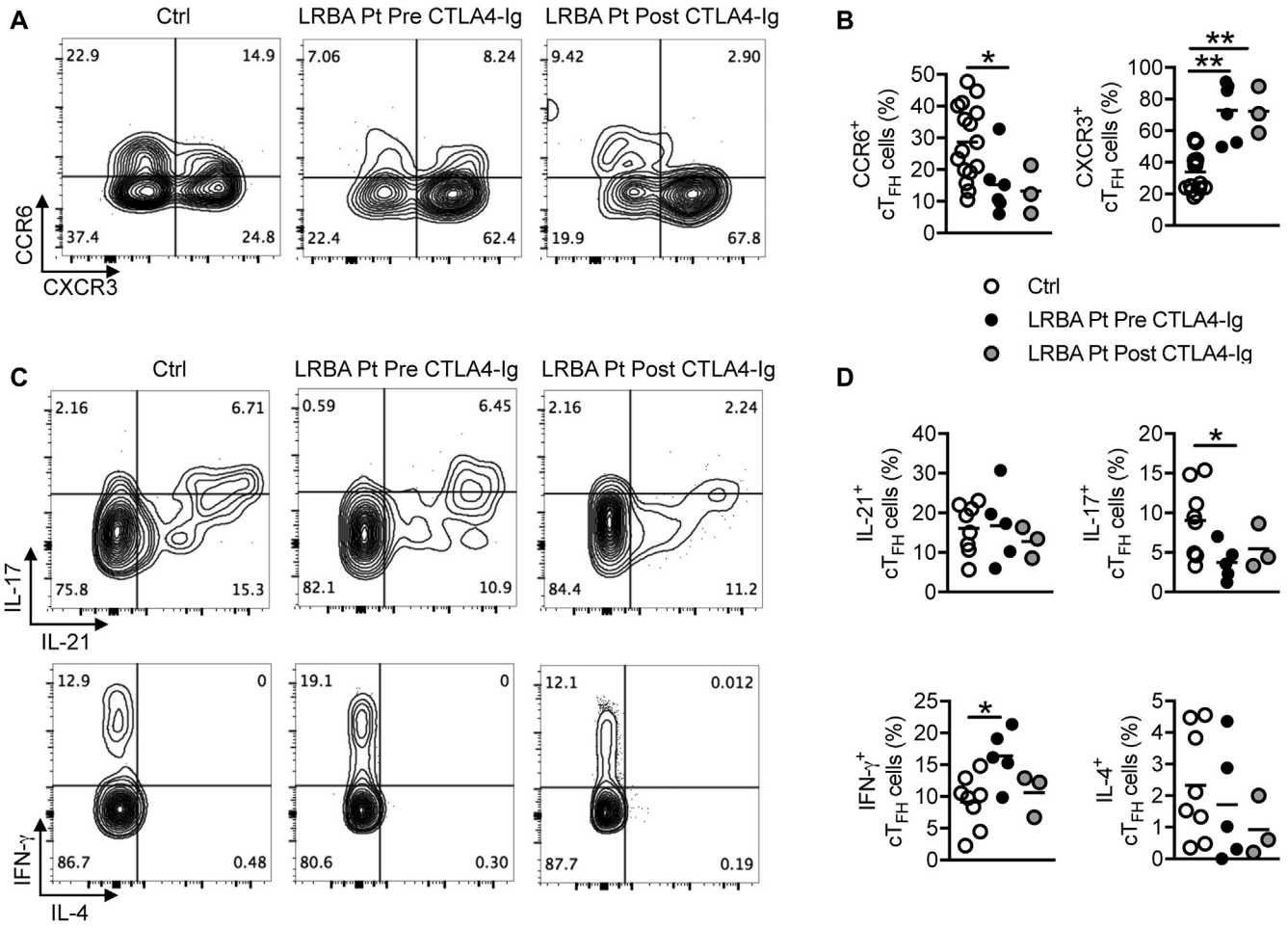

FIG 3. cT_{FH} cells of LRBA-deficient patients are skewed toward a T_H1-like cell phenotype. **A** and **B,** Flow cytometric analyses of CXCR3 and CCR6 expression in cT_{FH} cells of LRBA-sufficient and LRBA-deficient subjects before and after CTLA4-Ig treatment. **C** and **D,** Flow cytometric analyses of IL-17 and IL-21 expression (Fig 3, *C, upper panels*) or IFN-γ and IL-4 (Fig 3, *C, lower panels*) and the respective scatter plot representation (Fig 3, *D*) in cT_{FH} cells of LRBA-sufficient and LRBA-deficient subjects before and after CTLA4-Ig treatment. $*P < .05$ and $**P < .01$, 1-way ANOVA with posttest analysis.

equivalent in their capacity to differentiate into iT_{FH} cells that expressed similar levels of ICOS regardless of the CTLA4 status of naive $CD4^+$ cells (Fig 5, *A-C*). However, iT_{FH} cells generated from LRBA-deficient naive T cells exhibited greater expression of PD-1 compared with those from LRBA-sufficient naive T cells (Fig 5, *D* and *E*). Both iT_{FH} cell differentiation and expression of ICOS and PD-1 were partially inhibited on treatment of the differentiating T-cell cultures with CTLA4-Ig (Fig 5, *B-E*).

Given the normal differentiation of LRBA-deficient T cells into iT_{FH} cells, we analyzed further the capability of LRBA-sufficient and LRBA-deficient Treg cells to control iT_{FH} cell differentiation. Treg cells were isolated from patients and healthy control subjects by means of cell sorting of $CD4^+CD25^+CD127^{low}$ Treg cells. Equal numbers of Treg cells from patients and control subjects were added to an equal number of control naive $CD4^+$ cells that were stimulated with IL-12, IL-23, and TGF-β in the presence or absence of anti-CTLA4. LRBA-deficient Treg cells did not suppress iT_{FH} cell differentiation compared with control Treg cells, which is indicative of their impaired function (Fig 5, *F* and *G*).

Patients with LRBA deficiency frequently present with hypogammaglobulinemia and defective specific antibody titers, with the majority exhibiting B-cell dysfunction and reduced class-switched memory B-cell numbers.[31,36] Nevertheless, most of these patients have autoimmunity with increased levels of circulating autoantibodies.[29,31,36] To determine whether the production of circulating autoantibodies responded to CTLA4-Ig therapy, we screened our patients for serum autoantibodies before and at 2 time points after CTLA4-Ig therapy using an array of 84 autoantigens.[34] Also included in the analysis were 4 healthy subjects whose sera were used as negative controls and 2 positive control sera, including 1 patient with IPEX syndrome and another patient with SLE. Patients with LRBA exhibited a number of circulating IgG autoantibodies whose relative abundance significantly decreased after CTLA4-Ig therapy (Fig 6, *A*).

We next used *in vitro* coculture studies to examine the functional capacity of LRBA-deficient cT_{FH} cells to support IgM and IgG antibody production by naive B cells. $CD4^+CD25^-CD127^{High}PD1^+CXCR5^+$ cT_{FH} cells and $CD19^+IgD^+$

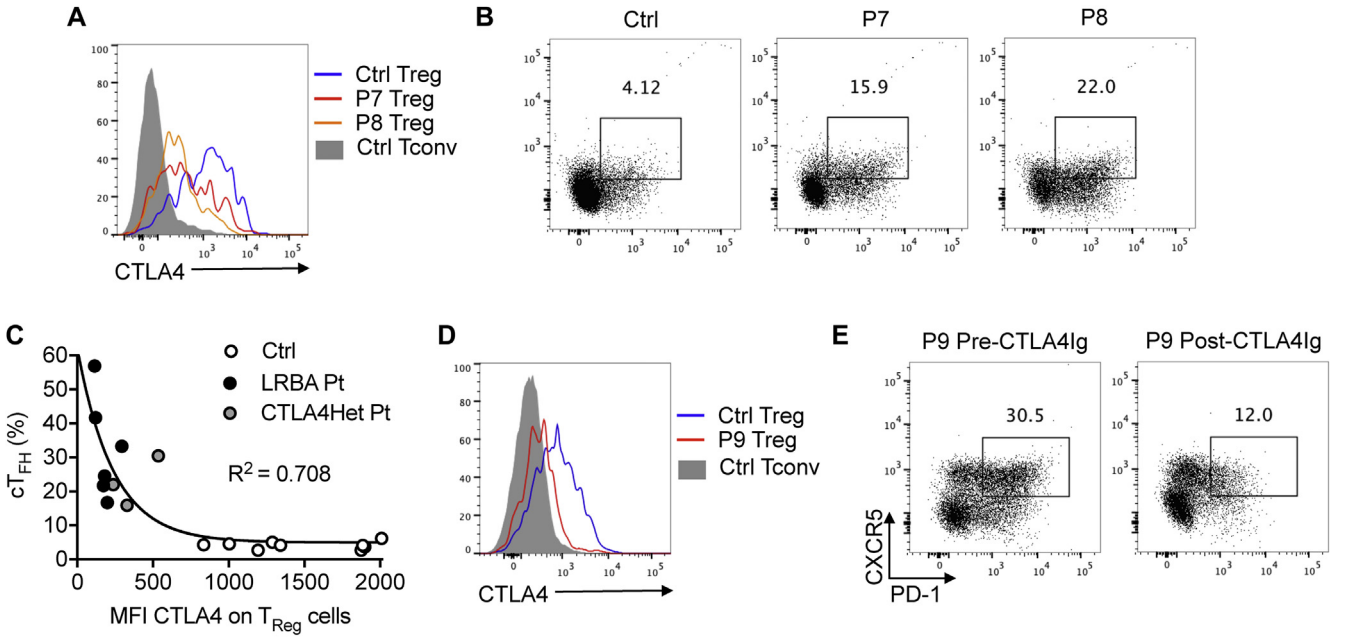

FIG 4. T_{FH} cell frequency in patients with CTLA4 deficiency and its response to CTLA4-Ig therapy. **A** and **B,** Flow cytometric analysis of CTLA4 expression on $CD4^+FOXP3^+$ Treg cells (Fig 4, *A*) and cT_{FH} cells (Fig 4, *B*) of control *(Ctrl)* subjects and patients P7 and P8 with a heterozygous CTLA4 mutation. **C,** Correlation between cT_{FH} cell frequencies and the mean fluorescence intensity *(MFI)* of CTLA4 expression in Treg cells of control (n = 8), LRBA-deficient (n = 6), and heterozygous CTLA4 mutant (n = 3) subjects. **D,** Flow cytometric analysis of CTLA4 expression on $CD4^+FOXP3^+$ cells in patient P9 with a heterozygous CTLA4 mutation. **E,** cT_{FH} cells in patient P9 before and after CTLA4-Ig therapy.

$CD27^-$ naive B cells were isolated from LRBA-deficient patient P3 and her fully HLA-matched healthy sister by means of cell sorting and cocultured in the presence of staphylococcal enterotoxin B. Results showed that although cT_{FH} cells of both the LRBA-deficient patient and her healthy sibling supported *in vitro* IgM and IgG antibody production by the sibling's naive LRBA-sufficient B cells (Fig 6, *B*), the LRBA-deficient cT_{FH} cells were more effective in that regard, which is consistent with their heightened activation state (Fig 2, *F* and *G*).[11,35] In contrast, the patient's cT_{FH} cells supported IgM but minimal IgG production by the patient's own B cells, which is in agreement with the previous report of defective IgG isotype switching in LRBA-deficient B cells.[26]

We also evaluated the effect of CTLA4-Ig treatment on B-cell phenotype. Although LRBA-deficient patients exhibited increased frequencies of naive (IgD^+CD27^{low}) and correspondingly decreased frequencies of switched memory (IgD^+CD27^{low}) B cells, CTLA4-Ig treatment did not change the ratio of naive/memory B cells in circulation (Fig 6, *C*). Given that expansion of T_{FH} cells promotes autoantibody production,[5-10] these findings indicated that the dysregulated T_{FH} cell expansion in patents with LRBA deficiency is functionally relevant to the excess autoantibody production in patients with this disorder.

DISCUSSION

Patients with deleterious mutations in the *LRBA* gene have dysregulated T_{FH} cell responses, as reflected by the high frequency of cT_{FH} cells, which might play a causative role in disease-related autoimmunity. In this report we examined the mechanisms of T_{FH} cell dysregulation in patients with LRBA deficiency and the usefulness of monitoring cT_{FH} cell frequencies as a measure of disease activity and response to therapy. We found that T_{FH} cell dysregulation involved failure of CTLA4-dependent Treg cells to control T_{FH} cell differentiation. Furthermore, the increased cT_{FH} cell frequencies in patients with LRBA deficiency and the related CTLA4 deficiency dramatically decreased after CTLA4-Ig therapy, which is in concordance with the decrease in other markers of disease activity and improved clinical outcome. Although cT_{FH} cell frequencies in LRBA-deficient subjects correlated tightly with other markers of immune dysregulation, such as sCD25, the former offers the advantage of ease of measurement by using flow cytometry and its sensitive and fast response to CTLA4-Ig therapy. Thus monitoring cT_{FH} cell frequencies in patients with LRBA and CTLA4 deficiencies might be particularly useful in tracking disease activity and response to different therapies.

Recent studies identified the critical role of CTLA4 in Treg cells in controlling humoral immunity. *In vivo* $Treg/T_{FR}$ cell depletion or selective deletion or blockage of CTLA4 in the Treg cell compartment resulted in unrestrained T_{FH} cell differentiation and profoundly increased autoantibody production.[21,22] In concordance with these studies, we demonstrated that LRBA-deficient naive $CD4^+$ T cells effectively differentiate into T_{FH} cells. However, LRBA-deficient Treg cells, like normal Treg cells treated with anti-CTLA4 mAb, did not suppress the expansion of *in vitro*–differentiated T_{FH} cells, pointing to the reduced CTLA4 expression on LRBA-deficient Treg cells as a key mechanism underlying the

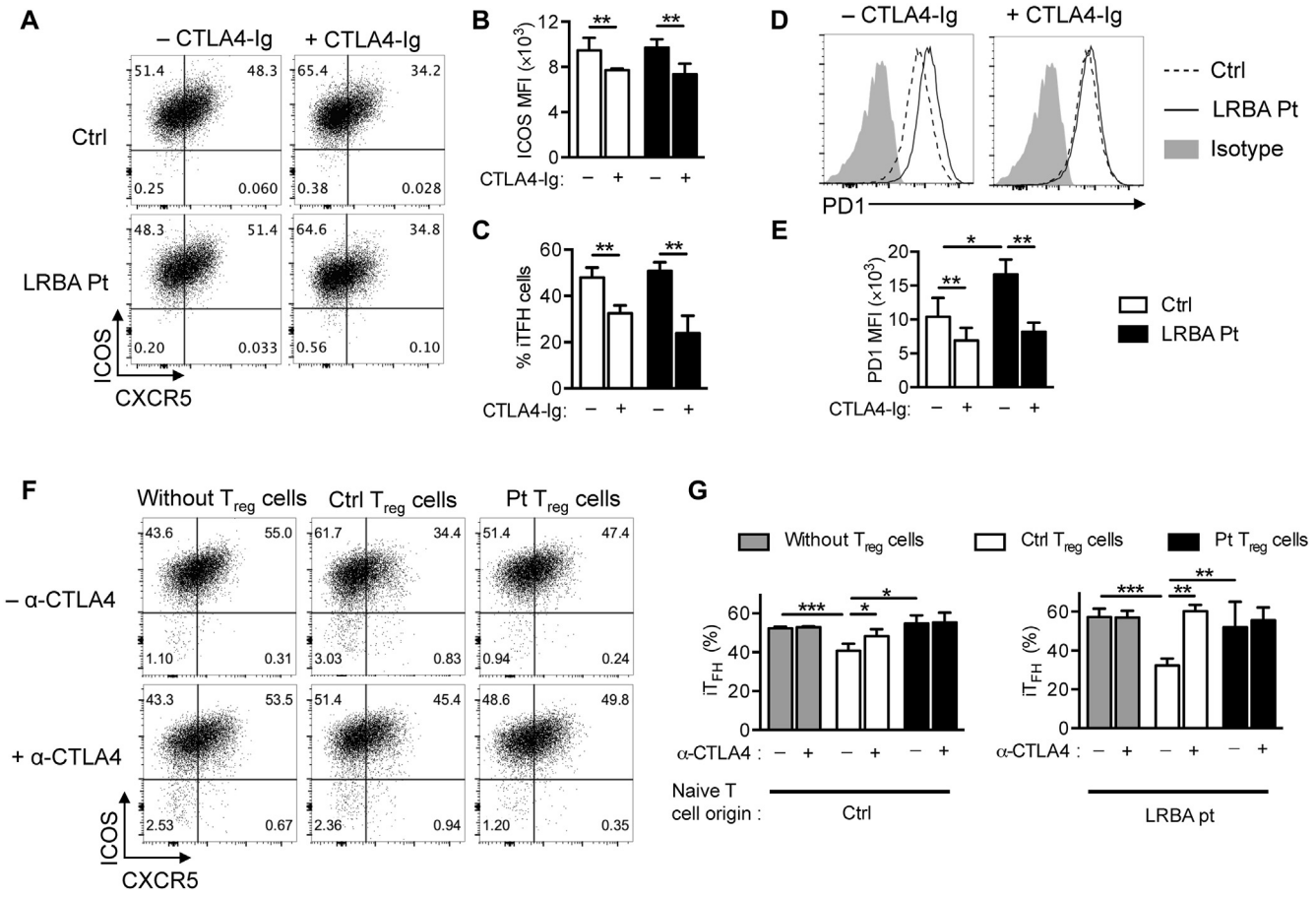

FIG 5. Ineffective Treg cell control of T_{FH} cell differentiation in LRBA-deficient subjects. **A,** Flow cytometric analysis of CXCR5 and ICOS expression in *in vitro*–differentiated iT_{FH}-like cells of control and LRBA-deficient subjects. **B** and **C,** Mean fluorescence intensity *(MFI)* of ICOS expression in iT_{FH}-like cells (Fig 5, *B*) and frequencies of *in vitro*–differentiated iT_{FH}-like cells (Fig 5, *C*) derived from control and LRBA-deficient naive $CD4^+$ T cells in the absence or presence of CTLA4-Ig. **D** and **E,** Flow cytometric analysis of PD-1 expression (Fig 5, *D*) and bar graph representation (Fig 5, *E*) of PD-1 expression in iT_{FH} cells of control and LRBA-deficient subjects. **F** and **G,** Flow cytometric analysis (Fig 5, *F*) and frequencies (Fig 5, *G*) of *in vitro*–differentiated iT_{FH}-like cells derived from control and LRBA-deficient naive $CD4^+$ T cells in the absence or presence of anti-CTLA4 mAb and/or Treg cells from patients and control subjects. Results are representative of 2 independent experiments. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$, 2-way ANOVA with posttest analysis.

dysregulated T_{FH} cell response in patients with LRBA deficiency.

Many patients with LRBA deficiency present with hypogammaglobulinemia and low numbers of switched memory B cells.[31,36] LRBA-deficient naive B cells have an intrinsic defect in their capacity to differentiate into IgM- and IgG-producing plasma cells.[26] Despite their B-cell switch defect, LRBA-deficient subjects mount an intense autoantibody response. cT_{FH} cells of LRBA-deficient subjects were activated, as evidenced by their heightened expression of ICOS and PD-1, a phenotype that was normalized on CTLA4-Ig therapy. They were more effective in supporting immunoglobulin production by LRBA-sufficient B cells compared with control cT_{FH} cells, which is indicative of their augmented functional capacity. *In vitro*–differentiated iT_{FH} cells also exhibited increased PD-1 expression that was reversed by CTLA4-Ig treatment, reflecting the role of CTLA4 deficiency in dysregulating LRBA-deficient T_{FH} cells both *in vitro* and *in vivo*. We suggest that the profound dysregulation of the T_{FH} cell compartment in LRBA-deficient subjects, precipitated by T_{FR} cell depletion and virtually absent CTLA4 expression, coupled with defective B-cell isotype switching and its associated somatic hypermutation might propel humoral autoimmunity by limiting the pruning of autoreactive antibodies normally achieved with somatic hypermutation.[37] Such a mechanism would be consistent with the reversal of T_{FH} cell dysregulation and downregulation of autoantibody production by CTLA4-Ig therapy. Because CD80 signaling favors B-cell isotype switching and immunoglobulin production, we cannot exclude an additional direct effect of CTLA4-Ig treatment on B cells, resulting in a reduction in autoantibody production in LRBA-deficient patients.[38] Further studies would be required to validate these predictions.

In summary, these findings support the monitoring of cT_{FH} cells as a sensitive marker for measuring the response of LRBA- and CTLA4-deficient patients to CTLA4-Ig therapy. Its use might help optimize the management of LRBA-deficient

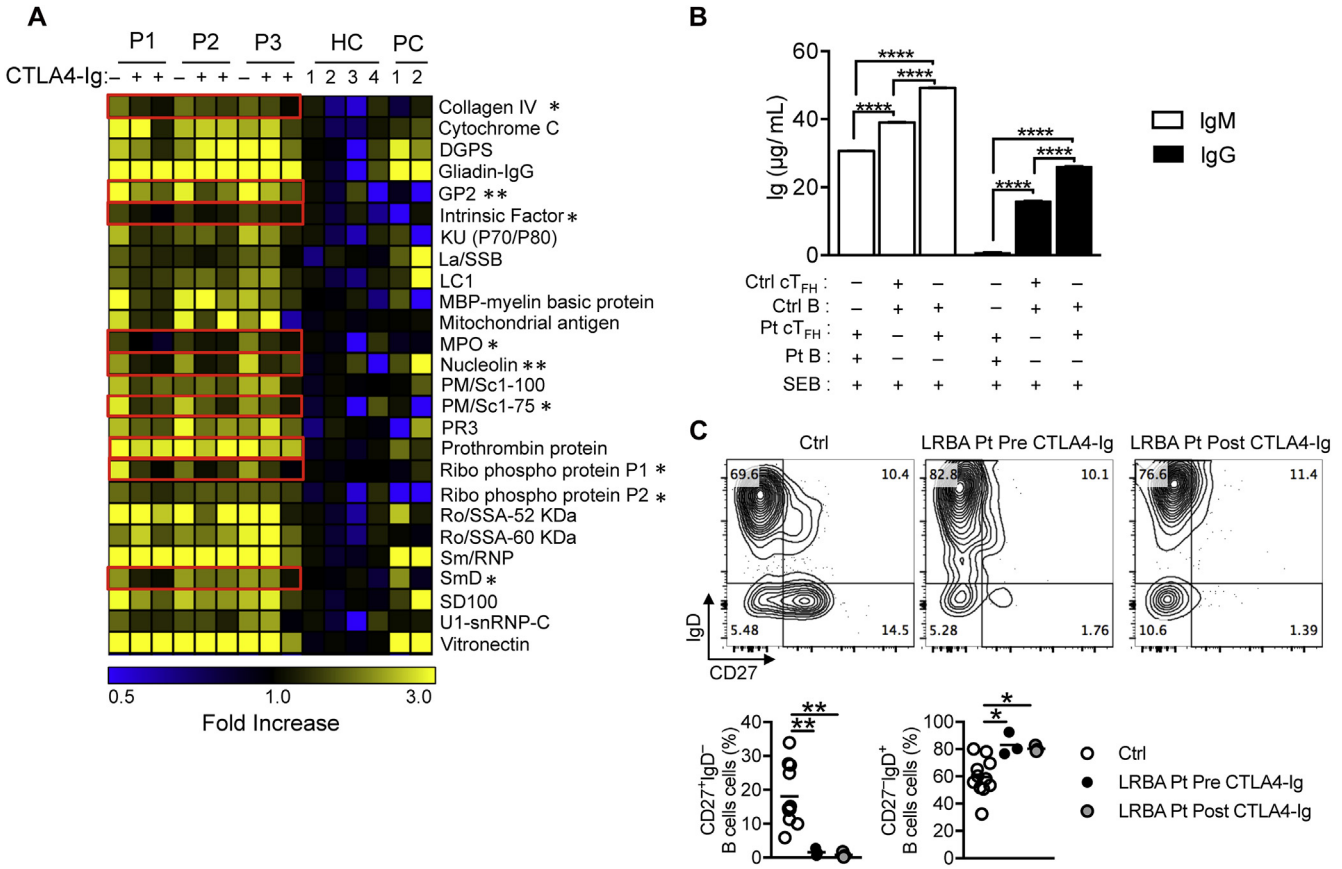

FIG 6. Autoantibody production and cT_{FH} cell function in LRBA-deficient subjects. **A,** Autoantibody production in LRBA-deficient patients before and after CTLA4-Ig therapy. Heat map showing IgG autoantibodies against self-antigens in sera of LRBA-deficient patients, healthy control subjects, patients with IPEX syndrome, and a patient with SLE. A value of 1 (black) is equal to the control average + 1 SD. Autoantibody responses affected by CTLA4-Ig therapy are boxed in red. **B,** IgM and IgG production in cocultures of cell-sorted cT_{FH} and naive B cells derived from patient P3 and her HLA fully matched sister in the presence of Staphylococcal enterotoxin B *(SEB)*. **C,** Flow cytometric analysis (Fig 6, *C, upper panels*) and scatter plot representation (Fig 6, *C, lower panels*) of CD27 and IgD expression on circulating B cells of control subjects and LRBA-deficient subjects before and after CTLA4-Ig treatment. **P* < .05 and ***P* < .01, Student unpaired 2-tailed *t* test; *****P* < .0001, 1-way ANOVA with posttest analysis.

patients to reach sustained clinical improvement and optimal preparation for hematopoietic stem cell transplantation. More broadly, monitoring the T_{FH}/T_{FR} cell axis might also aid in the diagnosis and treatment of primary immune dysregulatory disorders.

Key messages

- **LRBA and CTLA4 deficiencies result in highly increased frequencies of cT_{FH} cells.**
- **cT_{FH} cell dysregulation in patients with LRBA deficiency reflects impaired CTLA4-dependent suppression of T_{FH} cell differentiation by Treg cells.**
- **Monitoring cT_{FH} cell frequencies in patients with LRBA and CTLA4 deficiencies is useful in gauging the clinical response to CTLA4-Ig therapy.**

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METHODS

Case reports

Patient P3 is a 12-year-old girl, offspring of first-cousin parents from Saudi Arabia, who presented at the age of 2 years with autoimmune thrombocytopenia and Coombs-positive hemolytic anemia (Evan syndrome; Fig E1, *A*, and Table E1). She went on to have hepatosplenomegaly and lymphadenopathy. Over the next few years, she experienced complications of hemolytic anemia, including obstructive jaundice and cholelithiasis, for which she underwent splenectomy and cholecystectomy at the age of 6 years. Multiple immunosuppressive medications were tried without sustained clinical improvement, including steroids, rituximab, and mycophenolate mofetil. By 7 years of age, she started to have frequent oral candidal infections, as well as recurrent otitis media and pneumonia. Consequently, she had chronic lung disease with respiratory insufficiency obligating supplemental oxygen and prophylactic antibiotics (Table E1). Her initial chest computed tomographic scan showed a diffuse mixed ground-glass appearance with bilateral bronchiectasis and mediastinal adenopathy. Her lung biopsy revealed interstitial lymphoid hyperplasia, whereas lymph node pathology showed follicular hyperplasia with T-cell zone expansion. Her immunologic evaluation revealed low IgG and IgA and high IgM levels in the setting of low specific antibody responses (Table E2). Analysis of genetic defects leading to hyper-IgM syndrome did not show any deleterious mutations.

A diagnosis of common variable immunodeficiency was made, and intravenous immunoglobulin (IVIG) was started at that time. At 10 years of age, she was referred to the Boston Children's Hospital for further evaluation. She was given a diagnosis of LRBA deficiency caused by deletion of exon 57, as confirmed by cDNA sequencing and absent LRBA protein expression on flow cytometry and immunoblotting (Fig E1, *B-D*). Consistent with her LRBA deficiency, she was found to have profoundly altered Treg cell markers (Fig E1, *F*). The patient was started on weekly CTLA4-Ig (abatacept) infusion at 20 mg/kg with excellent clinical response (Fig E1, *E*). Her capillary oxygen saturation normalized, and she was taken off supplemental oxygen. Her platelet count initially increased to within the normal range for a few months but later decreased after tapering steroid therapy. Sirolimus was added to CTLA4-Ig, and the combination therapy enabled successful cessation of her steroid therapy with maintained clinical improvement. Follow-up immune studies revealed a steep decrease of her sCD25 levels, normalization of her serum IgM levels, and resolution of some, but not all, autoantibody responses. No changes were observed in serum IgA levels or in the circulating memory B-cell count, and the patient continued to require IVIG replacement therapy (Table E2).

Patient P4 is a 6-year-old Saudi girl, a product of consanguineous marriage, who presented at the age of 6 months with type 1 diabetes mellitus that responded well to insulin therapy and dietary restrictions. At 12 months of age, she started to have chronic diarrhea in the setting of negative workup for infectious agents. Multiple immunosuppressive medications, including pulse steroid, azathioprine, and tacrolimus, were tried without sustained clinical improvement. Subsequently, she started to have autoimmune thrombocytopenia at 5 years of age (Table E1). Evaluation of serum immunoglobulins, specific antibody titers, and lymphocyte subsets were normal apart from low IgA levels (Table E2). WES analysis showed a frameshift deletion mutation (c.4757_4760del, p.L1586fs) in the *LRBA* gene that leads to complete absence of LRBA expression (Fig E2, *A*). Flow cytometry revealed low CTLA4 expression on Treg cells and increased cT_{FH} cell frequency (Fig E2, *B-C*). she recently underwent hematopoietic stem cell transplantation from her fully matched healthy sibling.

Patient P5 is a 5½-year-old Turkish boy, a product of consanguineous marriage, who has had multiple admissions since the age of 9 months for recurrent fevers and intermittent nonbloody diarrhea. He was found to have hepatosplenomegaly and pancytopenia without a clear diagnosis (Table E1). His upper gastrointestinal endoscopy showed villous atrophy and increased intraepithelial lymphocyte counts. Despite a trial of a gluten-free diet, no clinical or histopathologic improvements were observed. Immunologic evaluation showed mild low IgM levels and normal lymphocyte subsets (Table E2). WES revealed a frameshift deletion mutation (c.7885delA, p.R2629fs) in *LRBA* confirmed by means of Sanger sequencing, which was associated with profoundly decreased LRBA expression (Fig E3, *A*). He was started on IVIG replacement therapy and CTLA4-Ig. After initiating those therapies, he experienced improvement in lymphoproliferation but continues to have chronic diarrhea despite increasing the CTLA4-Ig dose.

Patient P6 is a 12-year-old boy, brother of P5, who has had recurrent chest infections and chronic diarrhea since early infancy. At age 9 years, he was admitted to the hospital because of a persistent anal abscess in the setting of pancytopenia. He has had recurrent cytomegalovirus viremia in the past and started recently to have alopecia areata. Physical examination was notable for lymphoproliferation, which is similar to the clinical finding in his brother (Table E1). Although he was evaluated for immune dysregulation in the setting of a positive family history of LRBA deficiency, Sanger sequencing of *LRBA* identified the same frameshift mutation that was documented in P5 (c.7885delA, p.R2629fs), leading to decreased protein expression (Fig E3, *A*). Similar to other LRBA-deficient patients, P5 and P6 had low CTLA4 expression in Treg cells and increased cT_{FH} cell frequencies (Fig E3, *B* and *C*). Patient P6 was managed successfully with IVIG replacement and CTLA4-Ig therapy. Currently, his alopecia has resolved, and he has had marked improvement of his lymphoproliferation after 6 months of CTLA4-Ig therapy.

Patient P7 is a 26-year-old Turkish woman, a product of a first-cousin consanguineous marriage, who had recurrent upper respiratory tract infections and herpes labialis since the age of 6 years (Table E1). At the age of 24 years, she manifested hepatosplenomegaly, intra-abdominal lymphadenopathy revealed by using positron emission tomography, and unilateral tonsillar hypertrophy requiring tonsillectomy. At that time, EBV was detected in the blood at 2000 copies/mL, whereas histopathologic examination of the removed tonsillar tissue revealed EBV-encoded, small, RNA-positive, follicular-type lymphoid hyperplasia. At 25 years of age, she was hospitalized for jaundice and fatigue. Coombs-positive hemolytic anemia was identified, which was controlled with corticosteroid and high-dose IVIG and rituximab therapy. Her CTLA4 expression on Treg cells was decreased, whereas her cT_{FH} cell count was increased (Fig 4, *A* and *B*). Mutational analysis of her *CTLA4* genomic DNA revealed a heterozygous nonsense mutation in exon 2 of *CTLA4* (c.226C>T, p.Q76X; Fig E4). Currently, the patient is being followed up with subcutaneous immunoglobulin therapy, sirolimus, and azithromycin prophylaxis without new complications.

Patient P8 is the mother of patient P7, a 61-year-old woman who presented with recurrent sinusitis. There is no severe infection or hospitalization history. She has autoimmune hypothyroidism and high blood cytomegalovirus viral load (261,791 copies/mL; Table E1). Immunologic evaluation showed panhypogammaglobuinemia and mild CD4 lymphopenia (Table E3). Her CTLA4 expression on Treg cells was decreased, and her cT_{FH} cell counts were increased, which was similar to the condition of her daughter (Fig 4, *A* and *B*). She was found to have the same mutation as her daughter (Fig E4) and was started on subcutaneous immunoglobulin replacement therapy.

Patient P9 is a 17-year-old woman from the United States who presented at age 14 years with a persistent cough and sputum production (Table E1). Chest computed tomography showed diffuse pulmonary nodules and bronchiectasis in the right middle lobe and lingula. Lung biopsy revealed noncaseating granulomas. The patient's father had previously died of complications of multiple autoimmune diseases, including type I diabetes mellitus, autoimmune enteropathy, and autoimmune thyroid disease. On physical examination, the patient had a weight of less than the first percentile. She had no evidence of splenomegaly, hepatomegaly, or lymphadenopathy.

An immunologic evaluation revealed low IgA levels, T-cell lymphopenia, and detectable thyroid peroxidase autoantibodies in the context of a normal thyroid-stimulating hormone level (Table E3). Given the patient's granulomatous lung disease, selective IgA deficiency, and history of multiple autoimmune diseases in the patient's father, CTLA4 haploinsufficiency was considered. Her CTLA4 expression on Treg cells was dramatically decreased (Fig 4, *D*), whereas cT_{FH} cell frequency was markedly increased (Fig 4, *E*). Sanger sequencing of her *CTLA4* genomic DNA revealed a heterozygous mutation (c.356T>G, p. L119R; Fig E4), which was predicted to be pathogenic by

using Polyphen and SIFT protein function prediction algorithms. The patient was subsequently started on abatacept infusions at 20 mg/kg every 2 weeks with a good clinical response. The patient's cough and sputum production resolved. The percentage of cT_{FH} cells decreased after treatment (Fig 4, *E*). sCD25 levels, which were only mildly increased before abatacept treatment at 1228 U/mL, decreased to 750 U/mL after treatment. There was no change in IgA level. The patient continues on abetacept therapy and is currently clinically stable.

Antibodies

Anti-human mAbs to the following antigens were used for staining: CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD127 (A019D5), CD25 (BC96), CD45RA (HI100), CCR7 (G043H7), CXCR3 (G025H7), CCR6 (G034E3), IL-21 (3A3-N2), IL-4 (8D4-8), IFN-γ (4S.B3), IL-17 (BL168), IgD (IA6-2), CD19 (HIB19; BioLegend), CTLA4 (14D3), FOXP3 (PCH101), CXCR5 (MU5UBEE), PD-1 (eBioJ105), ICOS (D10.G4.1; eBioscience), and CD27 (M-T271; BD Biosciences) and the appropriate isotype controls.

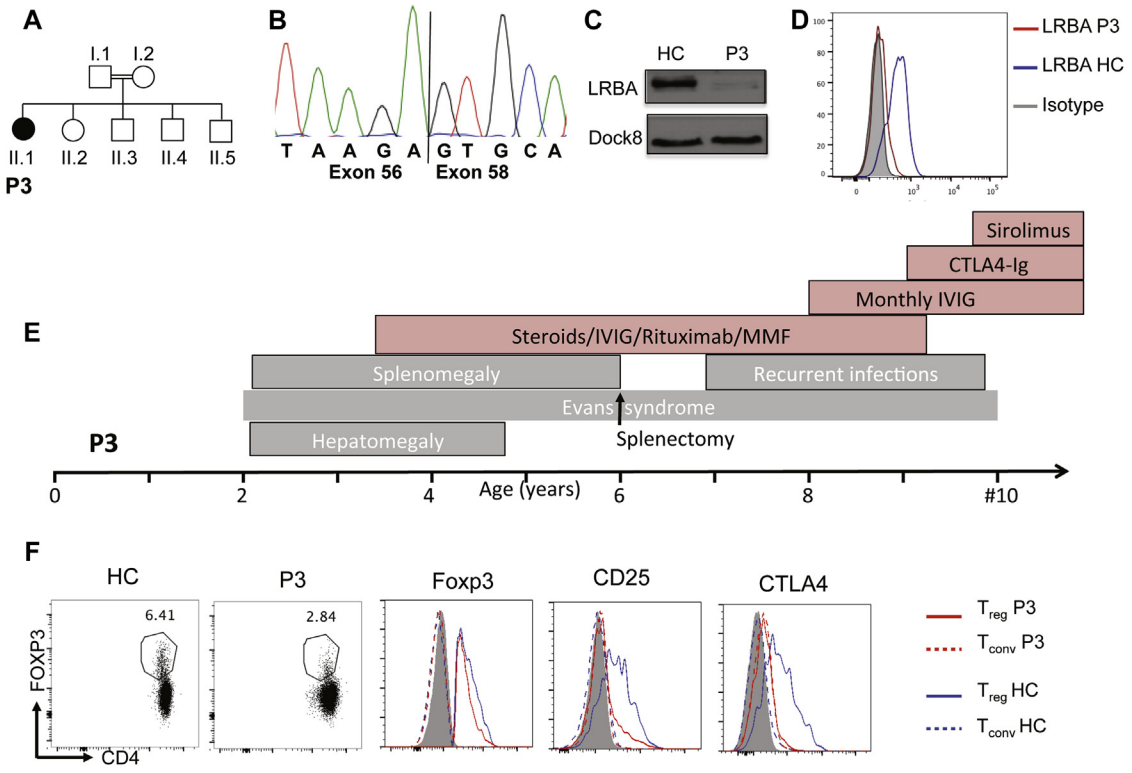

FIG E1. LRBA deficiency leads to profound immune dysregulation caused by defective Treg cell function. **A,** Pedigree of the LRBA-deficient patient P3. Generations are designated by Roman numerals (I-II), and subjects are designated by Arabic numerals. *Double lines* connecting parents indicate consanguinity. Probands in the respective families are indicated as II.1 to II.5. *Squares*, Male subjects; *circles*, female subjects; *solid symbols*, patient P3. **B,** Sanger sequencing analysis of *LRBA* cDNA in patient P3, showing deletion of exon 57. **C,** Western blot analysis of LRBA protein expression in lymphocytes of patient P3 compared with a healthy control subject. Blots were reprobed for dedicator of cytokinesis 8 as a positive control. **D,** LRBA expression in $CD3^+$ T lymphocytes from a control subject *(blue line)* and LRBA-deficient patient P3 *(red line)*. **E,** Timeline showing clinical features of LRBA deficiency in patient P3 and response to different kinds of therapy. **F,** Flow cytometric histogram plots of FOXP3, CD25, and CTLA4 expression in $CD4^+FOXP3^+$ T cells *(solid line)* and $CD4^+FOXP3^-$ T cells *(dotted line)* from a control subject *(blue line)* and LRBA-deficient patient P3 (red line). The *gray area* represents control isotype staining. *HC*, Healthy control subject.

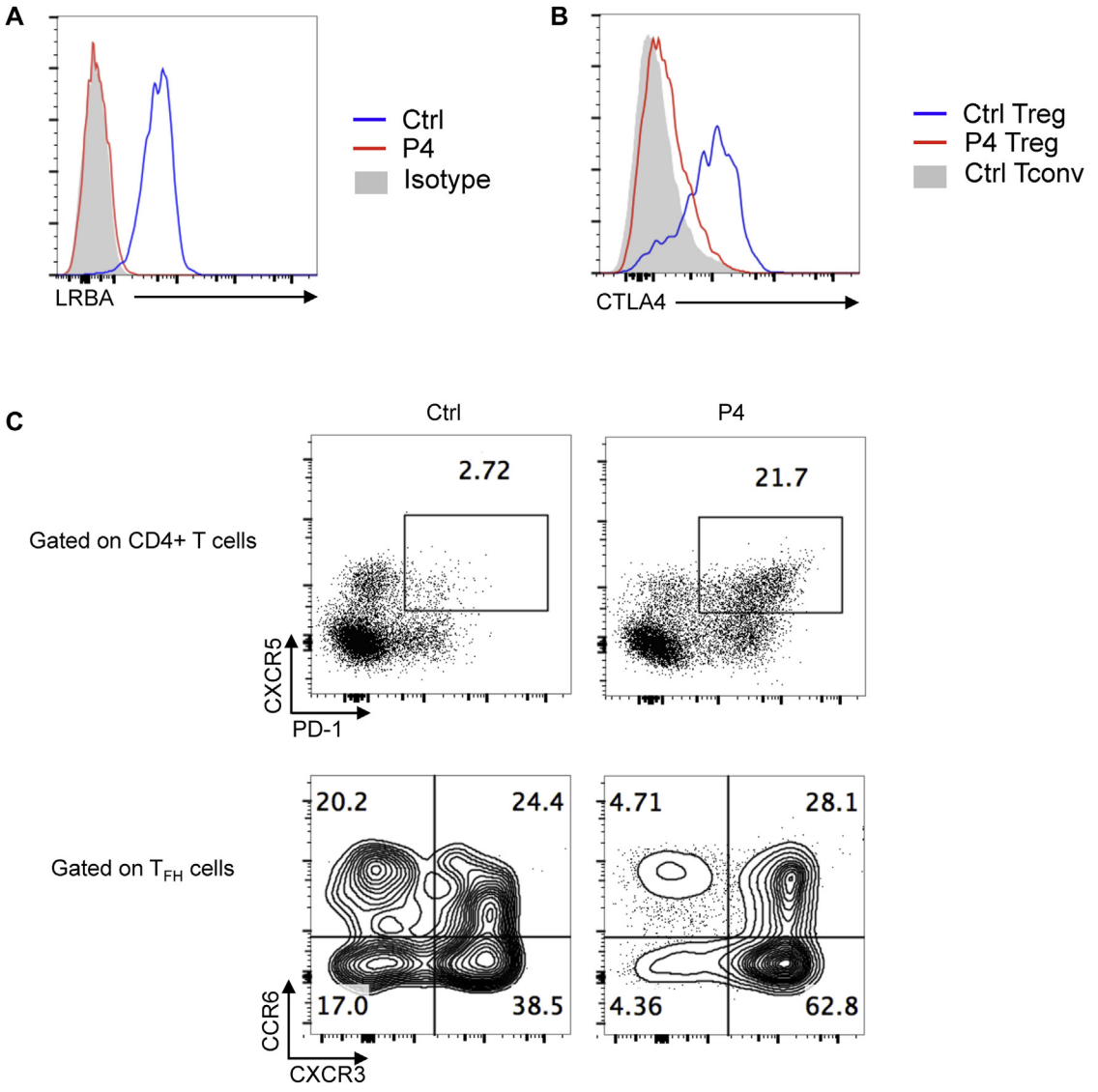

FIG E2. A, LRBA expression in PBMCs from control subjects *(blue line)* and LRBA-deficient patient P4 *(red line)*. The *gray area* represents control isotype staining. **B,** Flow cytometric histogram plots of CTLA4 expression in $CD4^+FOXP3^+$ T cells from a control subject *(blue line)* and LRBA-deficient patient P4 *(red line)*. The *gray area* represents $CD4^+FOXP3^-$ T cells from a control subject. *Tconv*, Conventional T cells. **C,** Flow cytometric analyses of CXCR5 and PD-1 expression in $CD4^+$ T cells *(upper panels)* and CXCR3 and CCR6 expression among $CXCR5^+PD\text{-}1^+CD4^+$ T cells (cT_{FH} cells; *lower panels*) in an LRBA-sufficient subject *(Ctrl)* and patient P4.

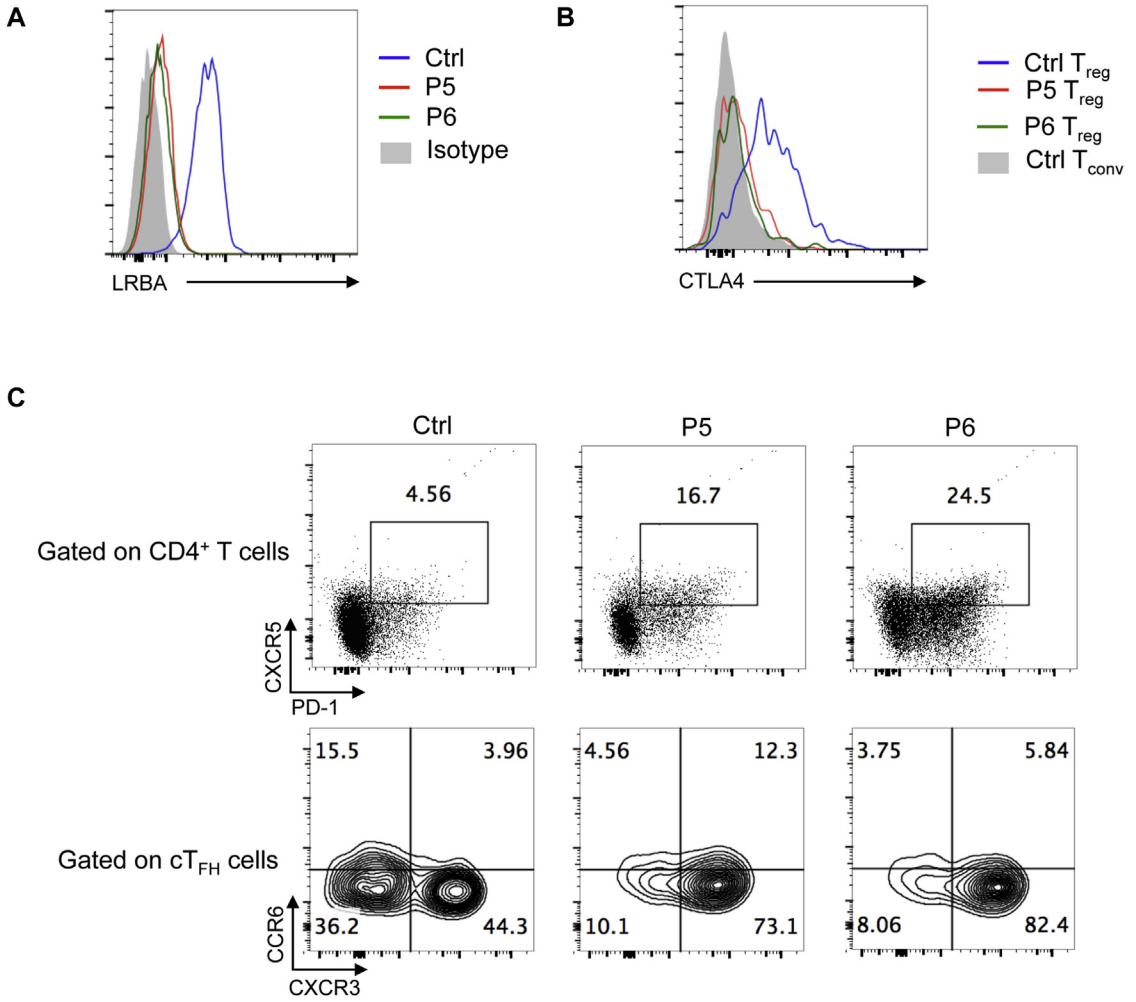

FIG E3. **A,** LRBA expression in PBMCs from control subjects *(blue line)* and LRBA-deficient patients P5 and P6 (*red line* and *green line*, respectively). The *gray area* represents control isotype staining. **B,** Flow cytometric histogram plots of CTLA4 expression in $CD4^+FOXP3^+$ T cells from a control subject *(blue line)* and LRBA-deficient patients P5 and P6 (*red line* and *green line*, respectively). The *gray area* represents $CD4^+FOXP3^-$ T cells from a control subject. *Tconv*, Conventional T cells. **C,** Flow cytometric analyses of CXCR5 and PD-1 expression in $CD4^+$ T cells *(upper panels)* and CXCR3 and CCR6 expression among $CXCR5^+PD\text{-}1^+CD4^+$ T cells (cT_{FH} cells; *lower panels*) in an LRBA-sufficient subject *(Ctrl)* and patients P5 and P6.

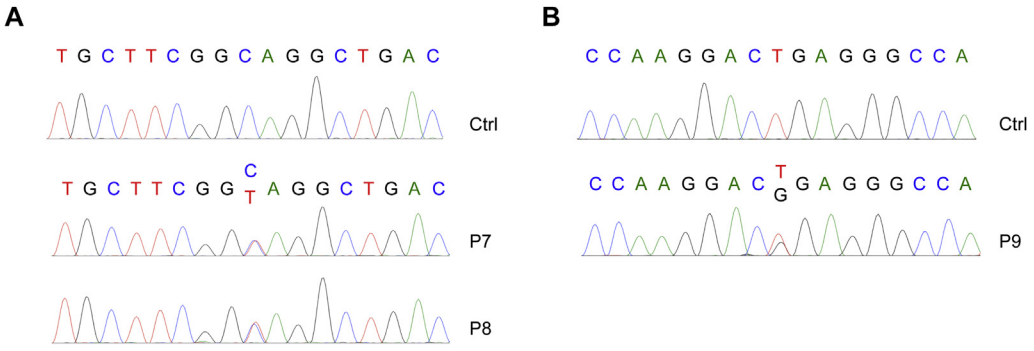

FIG E4. A, Sanger sequencing analysis of *CTLA4* DNA in patients P7 and P8, showing a heterozygous nonsense mutation in exon 2 (c.226C>T, p. Q76X). **B,** Sanger sequencing analysis of *CTLA4* DNA in patient P9, showing a heterozygous nonsense mutation in exon 2 (c.356T>G, p.L119R).

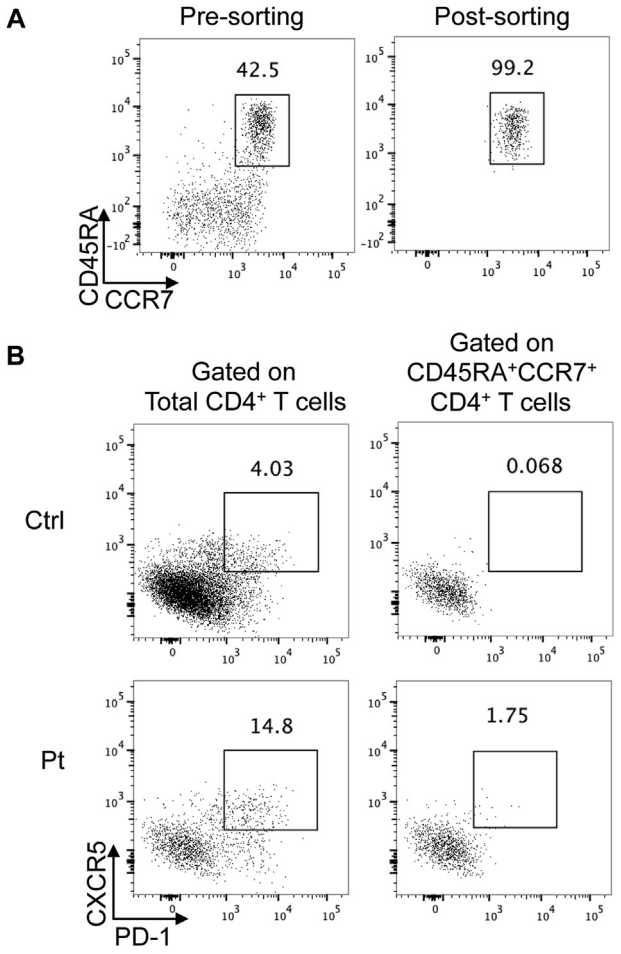

FIG E5. Sorting strategy of naive $CD4^+$ cells. **A,** Flow cytometric analysis of the purity of sorted naive $CD4^+$ ($CD45RA^+CCR7^+$) cells. **B,** Representative CXCR5 and PD-1 expression in total $CD4^+$ cells and cell-sorted naive $CD4^+$ cells of a control subject and LRBA-deficient patient.

TABLE E1. Clinical manifestations and management of LRBA- and CTLA4-deficient subjects

Patient	P1	P2	P3	P4	P5	P6	P7	P8	P9
Clinical data									
Sex/age (y)	Female/21	Female/18	Female/11	Female/6	Male/5	Male/12	Female/17	Female/26	Female/61
Sinopulmonary infections	+	+	+	−	−	+	+	+	+
Viral infections	+	+	+	−	+	+	−	+	+
Fungal infections	+	+	+	−	−	−	−	−	−
Mycobacterium species infection	−	−	−	−	−	−	−	−	−
Lymphoproliferation	+	+	+	–	+	+	+	+	−
Malignancies	−	−	−	−	−	−	−	−	−
Inflammatory brain lesion	−	+	−	−	−	−	−	−	−
Interstitial lung disease	+	+	+	−	−	−	+	−	−
AIHA	+	+	+	–	–	–	–	+	–
ITP	+	+	+	+	−	−	−	−	−
IDDM	−	+	−	+	−	−	−	−	−
Enteritis/colitis	+	+	−	+	+	+	−	−	−
Hepatitis	+	+	+	−	+	−	−	−	−
Thyroiditis	+	−	−	−	−	−	−	−	+
Medical and surgical therapy									
Steroid	+	+	+	+	−	−	−	+	−
Bactrim	+*	+*	+*	−	−	+*	−	+	+*
Azithromycin	+	+	+*	−	+*	−	+	+*	+
Immunoglobulin replacement	+*	+*	+*	−	+*	+*	−	+*	+*
Mycophenolate	−	−	+	−	−	−	−	−	−
Tacrolimus	+	+	−	+	−	−	−	−	−
Rituximab	+	+	+	−	−	−	−	+	−
Sirolimus	+	−	+*	−	−	−	+	+*	−
Azathioprine	−	−	−	+	−	−	−	−	−
Hydroxychloroquine	+*	+*	−	−	−	−	−	−	−
CTLA4-Ig	+*	+*	+*	−	+*	+*	+*	−	−
Splenectomy	−	−	+	−	−	−	−	−	−
HSCT	−	−	−	+	−	−	−	−	−

AIHA, Autoimmune hemolytic anemia; *HSCT*, hematopoietic stem cell transplantation; *IDDM*, insulin-dependent diabetes mellitus; *ITP*, idiopathic thrombocytopenic purpura.
*Ongoing medications.

TABLE E2. Hematologic and immunologic findings on LRBA-deficient subjects before and after CTLA4-Ig therapy

Patient	P1		P2		P3		P4	P5		P6		
CTLA4-Ig	Before	After	Before	After	Before	After	—	Before	After	Before	After	Normal range
Peripheral blood analysis												
WBC (cells/mL)	3.38	10.71	3.4	9.46	18.62	8.43	11.04	5.9	5.0	2.6	5.4	5,400-9,700
Hemoglobin (g/dL)	8.1	15.7	11.8	12.1	6.9	12.9	11.4	11.3	11.9	10.1	14.2	11.3-3.4
Platelets (cells/mL)	44	104	23	242	3	651	196	269	212	22	166	187,000-376,000
Neutrophils (cells/mL)	1.03	8.85	1.42	7.02	11.7	3.8	4.8	2.6	1.9	0.20	3.00	2,500-5,900
Lymphocytes (cells/mL)	1.42	1.3	1.49	1.72	4.6	3.5	5.3	2.8	2.8	1.7	1.8	1,230-2,760
Eosinophils (cells/mL)	0.79	0.02	0.05	0.10	1.12	0.43	0	0	0	0.1	0.1	40-1,900
Lymphocyte analysis												
CD3 (cells/mL)	1.87	1.85	0.27	1.48	7.02	2.76	5.86	2.02	2.01	1.19	1.20	1,000-2,600
CD3/CD4 (cells/mL)	1.21	0.99	0.22	0.80	3.03	0.79	2.93	0.97	1.17	0.76	0.67	530-1,500
CD4/CD45RA/CCR7$^-$ (%)	0.4	0.3	1	0.8	7.2	7.3	—	16.4	1.75	9.2	0.4	0.4-2.6
CD4/CD45RA/CCR7$^+$ (%)	11.4	9.9	1.6	19.1	7.8	20.6	—	36.8	74.8	33.8	55.3	57.1-84.9
CD4/CD45RO/CCR7$^-$ (%)	10.1	22.7	49.1	27.8	27.3	21.8	—	—	—	—	—	3.3-15.2
CD4/CD45RO/CCR7$^+$ (%)	78.1	67.1	49.1	52.3	57.8	50.3	—	—	—	—	—	11.3-26.7
CD3/CD8 (cells/mL)	0.59	0.83	0.42	0.63	0.35	0.17	2.32	0.85	0.70	0.38	0.41	330-1,100
CD8/CD45RA/CCR7$^-$ (%)	2.2	11.1	11.7	16	22.2	2.2	—	40.3	20.2	52.8	38.9	9.1-49.1
CD8/CD45RA/CCR7$^+$ (%)	28	22.3	9.2	34.3	4.2	6.3	—	29.8	43.2	21.45	35.6	28.4-80.6
CD8/CD45RO/CCR7$^-$ (%)	44.7	84.3	68	40.9	67.1	57.7	—	—	—	—	—	6.2-29.3
CD8/CD45RO/CCR7$^+$ (%)	25.1	18.3	11.1	8.9	6.5	6.4	—	—	—	—	—	1-4.5
CD3/CD4$^-$/CD8-TCR αβ	0.7	—	—	—	2.5	0.6	—	88.7	94.6	88.6	90.9	0.2-1.1
CD3/TCRγ δ%	2.5	—	—	—	10.7	11.4	—	7.7	4.6	10.6	8.2	0.6-12
CD4/CD25^{hi}/CD127^{lo} (%)	10.1	5.2	3.3	3.5	8.1	5.2	—	—	—	—	—	6.8-14.4
CD3/CD31/CD45RA	6.8	—	2	16.9			—	34	59	32.4	50.5	32.9-61.5
CD19 (cells/mL)	0.15	0.29	0.21	0.28	0.22	0.74	5.51	0.50	0.60	0.31	0.37	270-8,600
CD19/CD27$^-$/IgD$^+$ (%)	98.1	96.9	76.4	85.8	94.6	97.5	—	80.8	83.9	77.2	89	51.30-82.50
CD19/CD27$^+$/IgD− (%)	2.7	0.5	1.7	3.5	1	1.4	—	4.6	1.6	7.3	2.88	8.7-25.6
CD19/CD27$^+$/IgD$^+$ (%)	4.8	1.9	2.9	3.3	3.2	0.4	—	10.7	12.4	10.2	4.26	4.6-18.2
CD19/CD24^{hi}/CD38^{hi} (%)	0.4	0.3	2.8	4.5	2.5	39.1	—	—	—	—	—	7.2-23.8
CD19/CD24^{lo}/CD38^{hi} (%)	0.1	0.0	0.1	0.1	0.5	0.1	—	—	—	—	—	0.4-5.2
CD19/CD24^{hi}/CD38^{lo} (%)	15.4	13.1	9	25.5	4.5	2.4	—	—	—	—	—	12.6-36
CD19/CD21^{lo}/CD38^{lo}	42.2	8.8	75.5	21.2	75.40	20.5	—	5.9	1.08	20.7	4.05	1.1-11.9
CD16/CD56 (cells/mL)	0.13	0.14	0.04	0.07	0.95	0.22	1.22	0.20	0.12	0.17	0.15	70-480
Proliferation												
Concanavalin A (CPM)†	133	139	330	98	89	156	28	—	—	—	—	74,000-194,000
PHA (CPM)	160	120	55	101	105	182	80	—	—	—	—	104,000-319,000
Anti-CD3 (CPM)	144	139	39	109	58	120	—	—	—	—	—	78,000-205,000
Tetanus toxoid (CPM)	14	4	—	43	0.77	5.2	—	—	—	—	—	14,000-96,000
Candida albicans (CPM)	13.4	14.5	—	27.3	61.6	153	—	—	—	—	—	20,000-189,000
Immunoglobulin												
IgA (mg/dL)	<35	10	<2	28	16	<7	<5	70*	—	40*	—	70-312
IgG (mg/dL)	218*	1,190	419*	964	240*	901	243	1,710*	—	907*	—	639-1,344
IgM (mg/dL)	68	74	93	45	1,620	164	132	50*	—	90*	—	40-240
IgE (U/mL)	—	<1	22	<1	2	<1	—	39*	—	11.2*	—	0-500
Tetanus titer (IU/mL)	Low	—	Low	—	Low	—	Normal	—	—	—	—	>0.15
Pneumococcal titers (μg/mL)	Low	—	Low	—	Low	—	Normal	—	—	—	—	>1.3

*Quantitative determination of serum immunoglobulins before CTLA4-Ig therapy is reported for the respective patients just before starting IVIG therapy.
†CPM: counts per minute of ^{3}H-thymidine incorporated into DNA of proliferating T lymphocytes.

TABLE E3. Hematologic and Immunologic findings on CTLA4-deficient subjects

Patient	P7		P8	P9	
CTLA4-Ig	Before	After	—	—	Normal range
Peripheral blood analysis					
WBC (cells/mL)	3.96	5.37	4.1	5.4	5,400-9,700
Hemoglobin (g/dL)	9.7K	10.9	9.6	11.2	11.3-13.4
Platelets (cells/mL)	177	153	154	248	187,000-376,000
Neutrophils (cells/mL)	2.33	3.46	2.8	3.6	2,500-5,900
Lymphocytes (cells/mL)	0.86	1.02	1.10	1.10	1,230-2,760
Eosinophils (cells/mL)	0.39	0.44	0.06	0.10	40-1,900
Lymphocyte analysis					
CD3 (cells/mL)	0.67	0.83	0.57	0.80	1,000-2,600
CD3/CD4 (cells/mL)	0.52	0.63	0.16	0.29	530-1,500
CD4/CD45RA/CCR7$^-$ (%)	6.30	0.20	10	21	0.4-2.6
CD4/CD45RA/CCR7$^+$ (%)	11.1	20.3	24	6.0	57.1-84.9
CD4/CD45RO/CCR7$^-$ (%)	18.9	13.8	—	—	3.3-15.2
CD4/CD45RO/CCR7$^+$ (%)	69.8	65.7	—	—	11.3-26.7
CD3/CD8 (cells/mL)	0.12	0.17	0.39	0.52	330-1,100
CD8/CD45RA/CCR7$^-$ (%)	6.30	5.4	70	47	9.1-49.1
CD8/CD45RA/CCR7$^+$ (%)	65.4	62.0	6.30	3.00	28.4-80.6
CD8/CD45RO/CCR7$^-$ (%)	9.3	12.0	—	—	6.2-29.3
CD8/CD45RO/CCR7$^+$ (%)	19.0	20.6	—	—	1-4.5
CD3/CD4$^-$/CD8$^-$TCR αβ	—	—	1.40	0.70	0.2-1.1
CD3/TCR γδ (%)	—	—	2.10	1.20	0.6-12
CD4/CD25^{hi}/CD127^{lo} (%)	6.50	—	—	—	6.8-14.4
CD3/CD31/CD45RA	—	—	22	5.00	32.9-61.5
CD19 (cells/mL)	0.29	0.25	0	0.90	270-8,600
CD19/CD27$^-$/IgD$^+$ (%)	91.1	90.3	—	85.4	51.30-82.50
CD19/CD27$^+$/IgD$^-$ (%)	1.00	1.50	—	1.00	8.7-25.6
CD19/CD27$^+$/IgD$^+$ (%)	4.40	5.40	—	3.00	4.6-18.2
CD19/CD24^{hi}/CD38^{hi} (%)	7.90	15.9	—	3.60	7.2-23.8
CD19/CD24^{lo}/CD38^{hi} (%)	0	0	—	4.80	0.4-5.2
CD19/CD24^{hi}/CD38^{lo} (%)	28.7	29.1	—	—	5.3-18.9
CD19/CD21^{lo}/CD38^{lo} (%)	18.3	0.10	—	29	1.1-11.9
CD16/CD56 (cells/mL)	0.11	0.11	0.20	0.14	70-480
Proliferation					
PHA (CPM)†	133	—	—	—	104,000-319,000
Anti-CD3 (CPM)	47.0	—	—	—	78,000-205,000
Tetanus toxoid (CPM)	37.6	—	—	—	14,000-96,000
Candida albicans (CPM)	16.9	—	—	—	20,000-189,000
Immunoglobulin					
IgA (mg/dL)	<7*	<7	7*	6*	70-312
IgG (mg/dL)	1,252*	981	336*	847*	639-1,344
IgM (mg/dL)	64*	47	122*	20*	40-240
IgE (U/mL)	1*	1	1.4*	<1*	0-500
Tetanus titer (IU/mL)	Normal	Normal	—	—	>0.15
Pneumococcal titers (μg/mL)	Normal	Normal	—	—	>1.3

*The quantitative determination of serum immunoglobulins before CTLA4-Ig therapy is reported for the respective patients just before starting IVIG therapy.
†CPM: counts per minute of ^{3}H-thymidine incorporated into DNA of proliferating T lymphocytes.