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ORIGINAL ARTICLE

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Comparing the levels of CTLA-4-dependent biological defects in patients with LRBA deficiency and CTLA-4 insufficiency

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Abstract

Background: Lipopolysaccharide-responsive beige-like anchor protein (LRBA) deficiency and cytotoxic T-lymphocyte protein-4 (CTLA-4) insufficiency are recently described disorders that present with susceptibility to infections, autoimmunity, and lymphoproliferation. Clinical and immunological comparisons of the diseases with long-term follow-up have not been previously reported. We sought to compare the clinical and laboratory manifestations of both diseases and investigate the role of flow cytometry in predicting the genetic defect in patients with LRBA deficiency and CTLA-4 insufficiency.

Methods: Patients were evaluated clinically with laboratory assessments for lymphocyte subsets, T follicular helper cells (T_{FH}), LRBA expression, and expression of CD25, FOXP3, and CTLA4 in regulatory T cells (Tregs) at baseline and 16 h post-stimulation.

Results: LRBA-deficient patients ($n = 29$) showed significantly early age of symptom onset, higher rates of pneumonia, autoimmunity, chronic diarrhea, and failure to thrive compared to CTLA-4 insufficiency ($n = 12$). In total, 29 patients received abatacept with favorable responses and the overall survival probability was not different between transplanted versus non-transplanted patients in LRBA deficiency. Meanwhile, higher probability of survival was observed in CTLA-4-insufficient patients ($p = 0.04$). The T-cell subsets showed more deviation to memory cells in CTLA-4-insufficiency, accompanied by low percentages of Treg and dysregulated cT_{FH} cells response in both diseases. Cumulative numbers of autoimmunities positively correlated with cT_{FH} frequencies. Baseline CTLA-4 expression was significantly diminished in LRBA deficiency and CTLA-4 insufficiency, but significant induction in CTLA-4 was observed after short-term T-cell stimulation in LRBA deficiency and controls, while this elevation was less in CTLA-4 insufficiency, allowing to differentiate this disease from LRBA deficiency with high sensitivity (87.5%) and specificity (90%).

Conclusion: This cohort provided detailed clinical and laboratory comparisons for LRBA deficiency and CTLA-4 insufficiency. The flow cytometric approach is useful in predicting the defective gene; thus, targeted sequencing can be conducted to provide rapid diagnosis and treatment for these diseases impacting the CTLA-4 pathway.

KEYWORDS
CTLA-4, inborn errors of immunity, LRBA, T follicular helper cells, Treg

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GRAPHICAL ABSTRACT

LRBA deficiency and CTLA-4 insufficiency are immune dysregulatory disorders presenting with infections, autoimmunity, and lymphoproliferation. LRBA deficiency shows an early-onset, more severe clinical course than CTLA-4 insufficiency. A stepwise diagnostic algorithm including clinical and flow cytometric assessments is useful in predicting the defective gene; thus, targeted sequencing can be conducted to provide early diagnosis for these diseases impacting the CTLA-4 pathway.
Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; CTLA4+/-, CTLA-4 insufficiency; CM, central memory; cTFH, circulating T follicular helper cells; EM, effector memory; LRBA, lipopolysaccharide-responsive beige-like anchor; LRBA-/-, LRBA deficiency; RTI, respiratory tract infection; Treg, regulatory T cells; t-SNE, t-distributed stochastic neighbor embedding.

1 | INTRODUCTION

Inborn errors of immunity (IEI) is a group of inherited immune diseases presenting with recurrent infections, autoimmunity, autoinflammation, and/or malignancies.[1] Defective tolerance mechanisms lead to a subtype of IEI characterized by regulatory T cells (Treg) defects, which includes autosomal recessive lipopolysaccharide-responsive beige-like anchor (LRBA) deficiency (*LRBA*$^{-/-}$) and autosomal dominant cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) insufficiency (*CTLA4*$^{+/-}$).[2–4] One of the most prominent checkpoint inhibitors in the immune system is cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), also known as CD152, which plays a critical role in the maintenance of peripheral tolerance. CTLA-4 is constitutively expressed on fork-head box P3$^+$ (FOXP3) Treg cells and transiently expressed after activation in CD4$^+$ and CD8$^+$ T cells.[5] A minor portion of CTLA-4 protein is found at the plasma membrane; however, 90% of CTLA-4 is located intracellularly due to rapid internalization by clathrin-mediated endocytosis following T cells activation.[6,7] CTLA-4 competes with CD28 and shows a higher affinity for binding to CD80 (B7-1) and CD86 (B7-2) ligands expressed on antigen-presenting cells, resulting in removal of CD80/CD86 by transendocytosis and T-cell inhibition.[8] LRBA, a cytosolic protein, is expressed in all cell types and takes part in many cellular processes by controlling vesicular trafficking, signal transmission, and apoptosis.[9,10] More recently, the study of LRBA function revealed an essential role of this protein in the intracellular trafficking of CTLA-4 by preventing its lysosomal degradation and allowing for recycling to the cell surface.[11]

Human LRBA deficiency and CTLA-4 insufficiency were recently described IEI diseases, characterized by recurrent respiratory tract infections, lymphoproliferation, and immunodysregulation, manifesting as enteropathy, cytopenias, autoimmune disorders, and/or malignancies.[4,10] Detailed immunological analyses imparted that the patients had reduced Treg numbers with low CTLA-4 expression and defective suppressive capacity, which plays a key role in the pathogenesis of both diseases, explaining the phenotypic overlap between LRBA-deficient and CTLA-4-insufficient subjects.[4,12] Furthermore, uncontrolled increase in memory T and reduced memory B cells are observed in most patients of both diseases.[2,13–15] Due to impaired CTLA-4 expression, an increase in circulating T follicular helper cells (cT$_{FH}$, CD4$^+$ PD1$^+$ CXCR5$^+$) and soluble (s) CD25 levels were detected in the patients, which decline after abatacept (CTLA4-immunoglobulin fusion protein, CTLA4-Ig) targeted therapy.[16]

Recently, long-term follow-up studies were reported for LRBA deficiency by describing the efficacy of the conventional and targeted therapies (abatacept), and hematopoietic stem cell transplantation (HSCT).[13,17] Abatacept showed very-well controlled disease activity in most patients, especially for the lymphoproliferation and chronic diarrhea.[13] Similarly, in the Schwab et al[15] CTLA-4 insufficiency cohort, 11 of 14 patients had resolved symptoms related to enteropathy, granulomatous–lymphocytic interstitial lung disease, thrombocytopenia, and lymphoproliferation, and showed some improvement of optic neuritis. However, there is still a debate

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regarding the long-term use of immunosuppressants including abatacept versus conducting HSCT at an early stage in both diseases.

Despite the similarities in their severe clinical findings, a comprehensive comparison between the two disorders is necessary to help distinguish between diseases for an accurate early diagnosis. On the contrary, gene sequencing is indispensable for a definitive molecular diagnosis, but mutation analysis techniques are not routinely available everywhere and usually have a longer turnaround time, potentially causing diagnostic delay. For this reason, clinical and laboratory algorithms based on flow cytometric evaluation could be an easier, faster, and cheaper option to provide an early diagnosis, and thereby allowing treatments to be quickly initiated before the development of complications.

Herein, we aimed to compare clinical and laboratory features of LRBA deficiency and CTLA-4 insufficiency, and provide a diagnostic algorithm, highlighting the differences between both diseases that can be useful for early diagnosis and treatment.

2 | MATERIALS AND METHODS

This multicenter study included 29 $LRBA^{-/-}$ and 12 $CTLA4^{+/-}$ patients. Genetic diagnosis was made by next-generation (targeted or whole exome) sequencing, confirmed by Sanger sequencing, as described previously.[13,18,19] The patients were recruited from 16 different Pediatric Immunology centers located in Turkey. The study was approved by the Marmara University, School of Medicine Ethics Committee (09.2018.624), and written informed consent was obtained from all parents and adult patients.

2.1 | Study design, clinical and laboratory evaluations

During the study, a questionnaire, including demographic and clinical data (age at onset of symptoms, age at diagnosis, family history, past infections, autoimmunity, systems involved, and treatments) was filled for every patient. We specifically evaluated the detailed responses of abatacept and HSCT treatments. Blood samples from all the participating patients were sent to the Marmara University Pediatric Allergy and Immunology Center for immunological assessment, including deep lymphocyte subset analysis, cT_{FH} cell enumeration, intracellular LRBA staining, baseline and 16 h anti-CD2, anti-CD3, and anti-CD28 beads stimulated Tregs ($CD4^+$ $CD25^+$ $FOXP3^+$) with CTLA-4 expressions. The alterations in cTFH cell frequencies and plasma sCD25 levels by ELISA were analyzed during abatacept therapy in the patients. The detailed methods and used antibodies are provided in the Data S1 and Table S1.

2.2 | Flow cytometric analysis

Peripheral lymphocyte subset analyses and intracellular protein staining were performed by flow cytometry as described previously[13,18,20] and compared with age-matched healthy reference values.[21] The FOXP3 and CTLA-4 expressions were evaluated at diagnosis before starting abatacept treatment. The ratio of mean fluorescence intensity (MFI) for intracellular CTLA-4 protein was determined by dividing that raw CTLA-4 MFI in memory Tregs (mTreg) cells by the raw CTLA-4 MFI of unstimulated $CD4^+$ naive conventional T (nTcons, $CD45RA^+$ $FOXP3^-$) cells, used as an internal control that does not express CTLA-4 protein.[22] Patient samples were analyzed concurrently with two independent age-matched healthy control samples. Furthermore, CTLA-4 levels were measured also in other IEI patients without mutations in *LRBA* and *CTLA4* to compare the differences between the disorders. All stained cells were acquired with a Navios EX cytometer (Beckman Coulter) and analyzed with FlowJo™ (Single Cell Analysis Software v10–10.6.2), Kaluza Analysis Software (Version 2.1), and Cytobank software (Beckman Coulter). The details are provided in the Data S1.

2.3 | Statistical analysis

The data were presented as mean ± standard deviation (SD) or median with min-max, as indicated. Kolmogorov–Smirnov distribution test was conducted to determine the normal distribution. Fisher's exact test was used for the comparison of categorical values. Comparison between patient and control groups for continuous values was carried out with Student unpaired *t* test and 1-way ANOVA with Tukey post-test or Kruskal–Wallis with Dunn's post-test, as indicated. Paired *t* test were used for within group comparisons. Pearson's test was used for correlation analyses. Receiver-operating characteristic test was used to determine the sensitivity and specificity. Analysis of overall survival (OS) was done using the Kaplan–Meier method (log-rank test). Differences were considered significant at a $p < 0.05$. Statistical analysis was done using GraphPad Prism 9 (GraphPad Software Inc.).

3 | RESULTS

3.1 | Demographic and clinical characteristics of the patients

We enrolled 29 $LRBA^{-/-}$ and 12 $CTLA4^{+/-}$ patients to the study. The patients' demographic characteristics and their clinical phenotypes are presented in Tables 1 and 2.

There were 17 (58.6%) males and 12 (41.4%) females in the LRBA-deficient cohort. The median age of patients was 16 (min-max: 6–37) years. The mean age at onset of symptoms was 3.1 ± 3.9 years, while the delay time in diagnosis was 9.5 ± 8.4 years. Apart from two, there was consanguinity in 21 (91.3%) families. When the LRBA-deficient patients were evaluated according to their first clinical manifestations, 13 (45%) presented as common variable immunodeficiency (CVID), 10 (34%) as autoimmune lymphoproliferative syndrome (ALPS) and six (21%) as immune dysregulation, polyendocrinopathy, enteropathy, and

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TABLE 1 Demographic, clinical, and mutation findings of LRBA-deficient patients

Patient	Family	Age (years)/ gender	Consanguinity	AOO (months)	Phenotype	Clinical Diagnosis	Mutation (homozygous)	Survival
LM1	F1	15/M	+	18	RTI, CD, ID, LP	CVID	c.5047C>T, p.R1683*	Alive
LM2	F1	11/F	+	48	CD, ID, LP	ALPS	c.5047C>T, p.R1683*	Died
LM3	F2	9/M	+	8	RTI, CD, ID, LP	ALPS	c.7885delA, p.R2629fs	Alive
LM4	F2	15/M	+	1	RTI, CD, ID, LP	ALPS	c.7885delA, p.R2629fs	Alive
LM5	F3	29/M	+	3	RTI, CD, ID, LP	CVID	c.767+5_767+8delGTAT, p?	Alive
LM6	F4	11/M	+	7	RTI, CD, ID, LP	CVID	c.2599C>T, p.Q867*	Died
LM7	F5	6/F	+	8	RTI, ID	IPEX-like	c.5172-2A>G, intron 30	Alive
LM8	F6	16/M	+	6	RTI, CD, ID, LP	ALPS	c.1963C>T, p.R655*	Alive
LM9	F7	23/M	–	18	RTI, CD, ID, LP	CVID	c.2836_2839delGAAA, p.E946*	Alive
LM10	F8	37/F	+	84	RTI, CD, ID, LP	CVID	c.7238dupG, p.S2413Rfs*1	Died
LM11	F8	14/M	+	8	RTI, CD, ID, LP	ALPS	c.7238dupG, p.S2413Rfs*1	Died
LM12	F9	25/M	+	42	RTI, CD, ID, LP	ALPS	c.2818dupC, p.Q940fs	Alive
LM13	F10	22/M	–	66	RTI, CD, ID, LP	ALPS	c.1963C>T, p.R655*	Alive
LM14	F11	19/F	+	4	RTI, CD, ID	CVID	c.2735_2738delGGGT, p.T912*	Alive
LM15	F12	16/F	+	24	RTI, ID, LP	ALPS	c.3396-3397delAC, p.D975Yfs*15	Alive
LM16	F13	13/M	+	13	RTI, CD, ID, LP	IPEX-like	c.2496C>T, p.C832*	Alive
LM17	F13	14/M	+	60	RTI, ID, LP	IPEX-like	c.2496C>T, p.C832*	Alive
LM18	F14	18/F	+	18	RTI	CVID	c.5537C>T, p.S1846L	Alive
LM19	F15	23/F	+	6	RTI, CD, ID, LP	CVID	c.7976C>G, p.S2659*	Alive
LM20	F16	7/F	+	9	CD	IPEX-like	c.1496C>A, p.S499*	Alive
LM21	F17	15/F	+	9	CD, ID	IPEX-like	c.3549_3550insA, p.A1184Sfs*34	Alive
LM22	F17	6/M	+	48	RTI	CVID	c.3549_3550insA, p.A1184Sfs*34	Alive
LM23	F18	14/M	–	88	RTI, CD, LP	CVID	c.1963C>T, p.R655*	Alive
LM24	F18	17/F	–	180	ID, LP	ALPS	c.1963C>T, p.R655*	Alive
LM25	F19	18/F	+	60	RTI, CD, ID	CVID	c.6372del, p.F2124Lfs*29	Alive
LM26	F19	22/F	+	2	RTI, CD, ID, LP	IPEX-like	c.6372del, p.F2124Lfs*29	Alive
LM27	F20	18/M	+	160	RTI, ID, LP	CVID	c.5505delT, p.I1836fs	Alive
LM28	F21	36/M	+	6	RTI, CD, ID, LP	ALPS	c.6867delA, p.E2289Dfs*28	Alive
LM29	F22	11/M	+	100	RTI, ID	CVID	Exon 3–4 deletion	Alive

Abbreviations: ALPS, autoimmune lymphoproliferative syndrome; AOO, age of onset; CD, chronic diarrhea; CVID, common variable immunodeficiency; F, female; ID, immunodysregulation; IPEX, immunodysregulation, polyendocrinopathy, enteropathy, X-linked; LM, LRBA-mutant; LP, lymphoproliferation; M, male; RTI, respiratory tract infection.

*represents the premature stop codon.

X-linked like disease (IPEX-like). One (3%) asymptomatic patient (LRBA-mutant (LM) 22) was diagnosed during the family screening. At the end of the study, the follow-up period was 5.1 ± 2.3 years and 25 (86.2%) patients were alive, whereas four (13.8%) patients died (LM2 and LM6 after HSCT, and LM10 and LM11 due to the severe course of the disease without receiving HSCT). The probability of OS was 87.4% (Figure 1A). Patients LM23-29 were not previously reported, while the rest of the patients were described in our prior study.[13] The detailed histories of patients with updated follow-up for the previously reported subjects are provided in the Data S1 and Table S2. Twenty-three patients received abatacept treatment (LM1-7, LM9-10, LM12-22, LM25-27, and LM29). The median onset of abatacept therapy was 14.1 years (min-max: 1.5–35) with a median duration time as 30 months (min-max: 2.9–60) for maintenance dose of 10–15 mg/kg per month. All abatacept received patients showed alleviation in their symptoms and only in LM10 after 2 years, abatacept was stopped due to inadequate response. This patient passed away due to the severe respiratory

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TABLE 2 Demographic, clinical and mutation findings of CTLA-4-insufficient patients

Patient	Family	Age (y)/ gender	Consanguinity	AOO (months)	Phenotype	Clinical Diagnosis	Mutation (heterozygous)	Survival
CM1	F1	40/M	–	144	RTI, ID, LP	ALPS	c.436G>A, p.G146R	Alive
CM2	F1	11/M	–	48	RTI, ID, LP	ALPS	c.436G>A, p.G146R	Alive
CM3	F2	30/F	+	288	RTI, CD, ID, LP	CVID	c.226C>T, p.Q76*	Alive
CM4	F2	65/F	+	240	RTI	Asymptomatic	c.226C>T, p.Q76*	Alive
CM5	F3	9/M	–	6	CD, ID, LP	CVID	c.515C>A, p.S172*	Alive
CM6	F4	8/M	–	1	RTI, CD, ID	CVID	Exon 1–4 deletion	Alive
CM7	F5	14/F	+	152	RTI, CD, ID, LP	ALPS	c.294del, p.D99Mfs*14	Alive
CM8	F5	42/F	+	120	RTI, ID	CVID	c.294del, p.D99Mfs*14	Alive
CM9	F6	15/M	–	108	RTI, ID, LP	ALPS	c.60G>A, p.W20*	Alive
CM10	F7	15/M	–	101	ID	CVID	c.254G>A, p.C85Y	Alive
CM11	F7	17/M	–	8	RTI, ID, LP	CVID	c.254G>A, p.C85Y	Alive
CM12	F7	46/M	–	–	–	Asymptomatic	c.254G>A, p.C85Y	Alive

Abbreviations: ALPS, autoimmune lymphoproliferative syndrome; AOO, age of onset; CD, chronic diarrhea; CM, CTLA-4-mutant; CVID, common variable immunodeficiency; F, female; ID, immunodysregulation; LP, lymphoproliferation; M, male; RTI, respiratory tract infection.
*represents the premature stop codon.

failure at the age of 37 years (Data S1). Ten LRBA-deficient patients (LM2, LM5, LM6, LM8, LM9, LM12, LM13, LM18, LM21, and LM29) received HSCT with a median age of 14 years (min–max: 10–27), and 80% of them were alive. Of note, LM8 and LM13 were not on abatacept before transplantation, while the rest of patients received abatacept. The OS probability was similar between transplanted versus non-transplanted patients ($p = 0.290$, Figure S1A). Apart from LM13, all transplanted patients have been followed up without medication, whereas all non-transplanted patients are still on IgRT and/or immunosuppressants to control disease activity.

The CTLA-4-insufficient patients were comprised of eight (66.7%) males and four (33.3%) females. The median age of patients was 16 (min–max: 8–65) years and the mean age at onset of symptoms was 9.2 ± 7.8 years, significantly higher than LRBA-deficient patients ($p = 0.030$). Consanguinity was noted in two families (28.5%), which was significantly less than in LRBA deficiency ($p = 0.002$). In total, six (50.0%) patients presented as CVID and four patients (33.3%) with ALPS phenotype (Table 2). Two (16.7%) asymptomatic patients (CTLA4-mutant (CM)4 and CM12) were diagnosed during family screening. All the patients were alive (100%) without HSCT, and the probability of OS was higher compared to LRBA deficiency ($p = 0.04$, Figure 1A). CM1, CM3, and CM5-8 received abatacept and showed good responses at maintenance dose of 10–15 mg/kg per month (Data S1 and Table S3). The follow-up period was 6.8 ± 8.1 years, which was similar to LRBA-deficient patients.

The pathogenic variants and affected domains of the genes are shown in Figure S1(B,C). Frameshift homozygous mutations in LM25, LM26, LM27, and LM28 were novel and not previously reported. The heterozygous mutations in *CTLA4* were mostly located in exon 2, including missense and nonsense changes. CM6 had a large heterozygous deletion encompassing exon 1–4. The Family 2 with the stop gain mutation was reported previously.[16]

3.2 | Clinical phenotypes of LRBA deficiency and CTLA-4 insufficiency

The three major clinical phenotypes among patients with LRBA deficiency were recurrent infections ($n = 25$, 86.2%), immunodysregulation (ID) ($n = 25$, 86.2%), and lymphoproliferation (LP) ($n = 21$, 72.4%), whereas CTLA-4 insufficiency presented with ID ($n = 10$, 83.3%), recurrent infections ($n = 9$, 75.0%), and LP ($n = 7$, 58.3%) (Figure 1B). There were no differences between the two diseases in terms of the major clinical symptoms. The detailed clinical symptoms and treatment modalities, including targeted therapies of patients, are presented in Tables S2 and S3, and Data S1.

The most prominent ID features in LRBA deficiency were chronic diarrhea ($n = 21$, 72.4%), immune thrombocytopenic purpura ($n = 13$, 44.8%), and autoimmune hemolytic anemia ($n = 12$, 41.4%). While in CTLA-4 insufficiency, the most notable features of ID were autoimmune hemolytic anemia ($n = 7$, 58.3%), chronic diarrhea ($n = 4$, 33.3%), immune thrombocytopenic purpura ($n = 4$, 33.3%), and hashimoto thyroiditis ($n = 4$, 33.3%). Chronic diarrhea was more commonly observed in LRBA deficiency ($p = 0.030$) than CTLA-4 insufficiency. Overall, cumulative numbers of autoimmunities were higher in LRBA deficiency ($p = 0.040$, Figure S2A). Furthermore, malignancies including Hodgkin lymphoma (LM15) and gastric adenocarcinoma (LM5) were observed only in LRBA cohort, as reported in our previous study.[13]

When the respiratory features were compared between two disorders (Figure 1C), the frequency of pneumonia was found to be significantly higher ($p = 0.036$) in LRBA-deficient patients ($n = 22$, 75.9% vs $n = 5$, 41.7%, respectively). Likewise, LRBA deficiency showed a more severe phenotype as reflected by failure to thrive (FTT) ($n = 17$ (58.6%) vs $n = 2$ (16.7%)) and digital clubbing ($n = 8$, 27.6% vs none) ($p = 0.031$, $p = 0.043$, respectively). Allergic manifestations like asthma and rhinitis were observed in 4 (14%)

LRBA-deficient and 5 (42%) CTLA-4-insufficient patients. Viral infections including cytomegalovirus (27.6% vs 25%), Epstein–Barr virus-EBV (17.2% vs 8.3%), varicella (20.7% vs 8.3%), and COVID-19 (17.7% vs 16.6%) were observed equally in both disorders. All the COVID-19-infected patients experienced only upper respiratory tract symptoms.

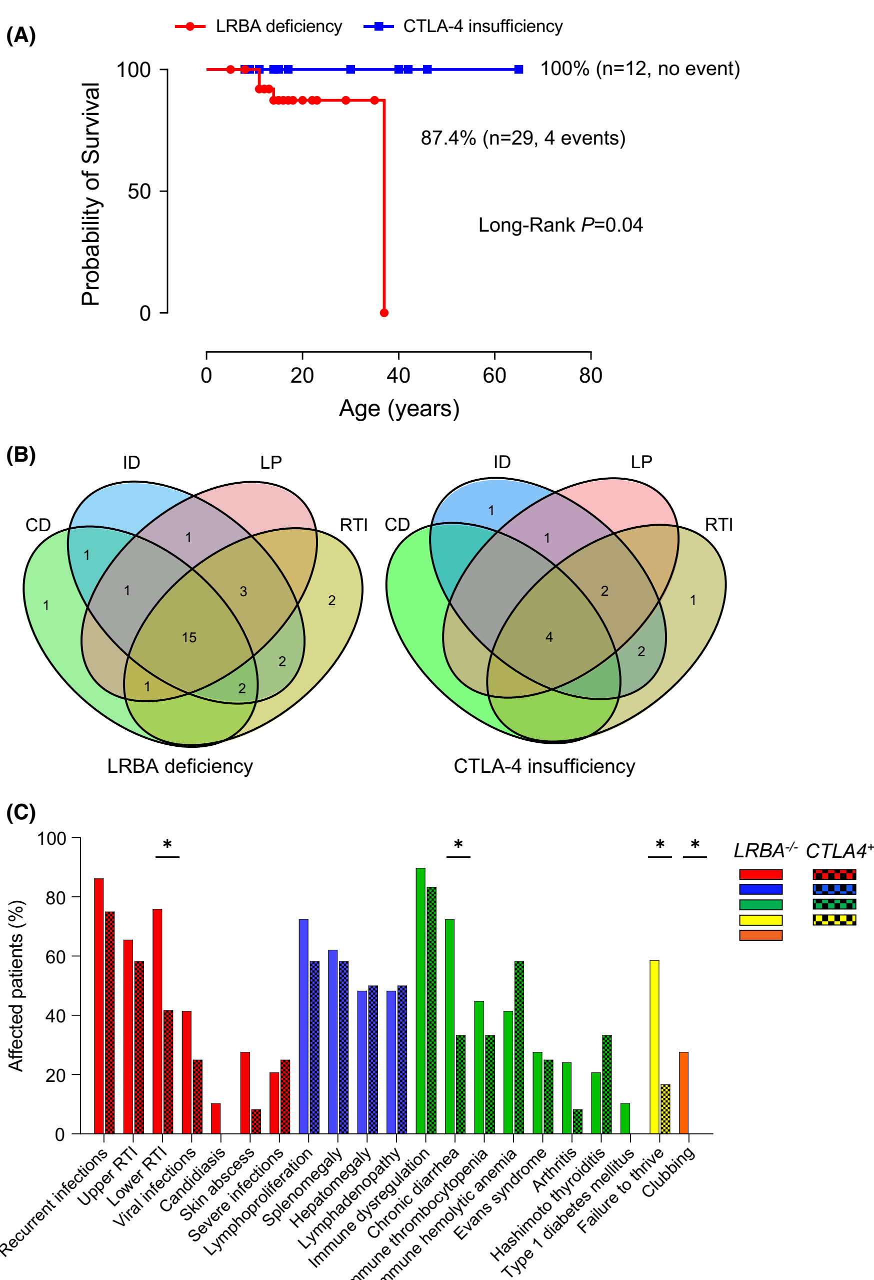

FIGURE 1 Clinical findings of LRBA deficiency and CTLA-4 insufficiency. (A) A Kaplan–Meier plot showing overall survival versus time (years). (B) Venn diagrams presenting the phenotype of patients characterized by chronic diarrhea (CD), immune dysregulation (ID), lymphoproliferation (LP), and respiratory tract infection (RTI). (C) Colored bars demonstrate the prevalence of the clinical parameters in LRBA- and CTLA-4-deficient patients. *$LRBA^{-/-}$*, LRBA deficiency; *$CTLA4^{+/-}$*, CTLA-4 insufficiency. *$p < 0.05$, Fisher's exact test

FIGURE 2 Flow cytometry analysis of peripheral blood in LRBA-deficient and CTLA-4-insufficient patients demonstrates abnormalities in T, B, and NK cells. (A) Absolute numbers were evaluated for total $CD3^+$, $CD4^+$, $CD8^+$ T, B, and NK cells, while the others were based on percentage. The heatmap shows the cells proportion of patients as high, low, or normal values according to the healthy age-matched reference values for the indicated parameters. White square indicates the unavailable data. (B) Representative t-distributed stochastic neighbor embedding (t-SNE) visualization of $CD4^+$ and $CD8^+$ T-cell subsets of patients with different colors, showing more memory skewing in CTLA-4 insufficiency. (C) Representative sunburst visualization of the percentage of B-cell subsets in patients and age-matched healthy control. LM, LRBA-mutant; CM, CTLA4-mutant; *$LRBA^{-/-}$*, LRBA deficiency; *$CTLA4^{+/-}$*, CTLA-4 insufficiency; EM, effector memory; EX, exhausted memory; RTE, recent thymic emigrants; DNT, double-negative T; CS, class-switched; NK, natural killer. Naïve T cells (Tn, $CD45RA^+CCR7^+$), central memory T cells (Tcm, $CD45RA^-CCR7^+$), effector memory T cells (Tem, $CD45RA^-CCR7^-$), terminally differentiated effector memory T cells (Tex, $CD45RA^+CCR7^-$), naive mature B cells ($CD19^+CD27^-IgD^+$), non-switched memory B cells ($CD19^+CD27^+IgD^+$), switched memory B cells ($CD19^+CD27^+IgD^-$). The statistical differences are indicated in the result section

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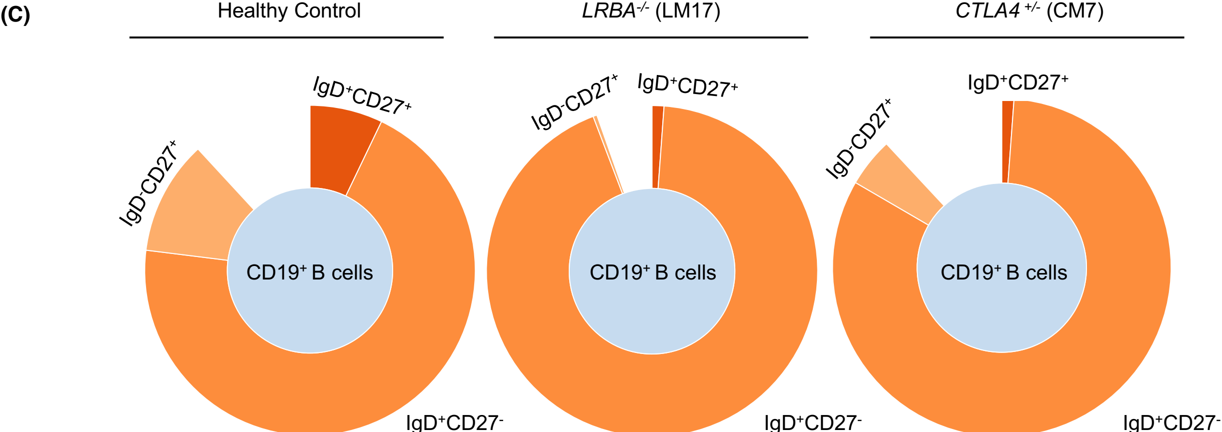
3.3 | Immunological phenotypes of LRBA deficiency and CTLA-4 insufficiency

Serum immunoglobulin levels before intravenous immunoglobulin therapy were available for 28 LRBA- and 11 CTLA-4-insufficient patients, and showed low IgG in 15 (53.5%), low IgM in 15 (53.5%), and low IgA in 23 (82.1%) LRBA deficiency patients, which was proportionally similar to CTLA-4-insufficient patients with low IgG in seven (64%), low IgM in six (55%), and low IgA in nine (82%). Extensive flow cytometric analysis at diagnosis including T, B,



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natural killer (NK), T-cell, and B-cell subtypes was performed and compared between the two diseases (Figure 2A, Tables S4 and S5). Interestingly, in *CTLA4*$^{+/-}$ patients, the normalized T-cell compartment according to the age-matched healthy controls reference values[21] demonstrated more skewing toward memory cells, characterized by diminished naive CD4^{+} T cells (CD45RA^{+}CCR7^{+}) and central memory CD4^{+} T cells (CD45RA^{-} CCR7^{+}) ($n = 11$, 91.7% and $n = 10$, 83.3%) compared to *LRBA*$^{-/-}$ patients ($n = 12$, 48.0% and $n = 7$, 28.0%) ($p = 0.040$, $p = 0.030$, respectively). Additionally, effector memory CD4^{+} T cells (CD45RA^{-} CCR7^{-}) were significantly higher in *CTLA4*$^{+/-}$ patients ($n = 11$, 91.7%, $p = 0.010$). The same dysregulated phenotype was also observed in CD8^{+} T-cell subgroups as central memory CD8^{+} T cells (CD45RA^{-} CCR7^{+}) were significantly reduced in *CTLA4*$^{+/-}$ samples ($n = 5$, 41.6%, $p = 0.002$). The phenotypic composition of cells was further evaluated by visualization tools developed to manage high-dimensional data (t-distributed stochastic neighbor embedding (t-SNE)), allowing a 2-dimensional visualization whereby phenotypically similar cells form a cluster. The analysis clearly revealed skewing toward memory T cells in CTLA-4 insufficiency (Figure 2B). There were no differences on B- and NK-cell subtypes between the two diseases, and both disorders showed low percentage of memory B cells compared to the age-matched healthy controls (Figure 2C).

Since CTLA-4 is important for controlling cT_{FH} cells,[16] we evaluated the frequency of cT_{FH} in both diseases. Baseline percentage of cT_{FH} cells was found to be higher in patients compared to the healthy matched controls (Figure 3A,B). Furthermore, cT_{FH} cells from patients exhibited more activation, measured by programmed cell death-1 (PD-1) expression when compared to healthy controls (Figure 3C). Meanwhile, cumulative numbers of autoimmunities positively correlated with cT_{FH} frequencies, prominently in *LRBA*$^{-/-}$ patients ($r = 0.660$, $p = 0.004$). We observed a significant reduction in cT_{FH} cells and PD-1 expression in LRBA deficiency and CTLA-4 insufficiency on abatacept treatment (Figure 3D,E). The normalization with abatacept treatment was also accompanied by a reduction in sCD25 in plasma samples of LRBA deficiency (Figure S2B). LRBA-deficient patients showed lower LRBA protein expression when compared to CTLA-4-insufficient patients and healthy controls (Figure S2C). Finally, we used our previously reported cutoff value of 0.76 for the quantified LRBA-MFI ratio,[13] which was able to identify all LRBA-deficient patients with a high sensitivity (100%) and specificity (91.7%) (Figure S2D).

3.4 | Reduced Treg cells in both LRBA deficiency and CTLA-4 insufficiency

The percentage of CD25^{+} FOXP3^{+} Treg cells gated on CD3^{+} CD4^{+} T cells in patients during diagnosis and healthy controls was evaluated at baseline (unstimulated) and after a brief stimulation for 16 h. At baseline, we detected a significant difference between patients and healthy controls (3.6 ± 1.2%) ($p = 0.0001$ for *LRBA*$^{-/-}$, $p = 0.003$ for *CTLA4*$^{+/-}$) with no significant differences between *LRBA*$^{-/-}$ (1.5 ± 1.0%) and *CTLA4*$^{+/-}$ (1.6 ± 1.2%) patients (Figure 4A,B). A significant induction of Treg cells was observed after stimulation in both groups of patients ($p < 0.0001$ and $p = 0.003$, respectively), however; the levels still low compared to age-matched controls (Figure 4B and Figure S3A–C). The reduced Treg cells were accompanied by decreased canonical markers, including CD25 (IL-2RA) and FOXP3, which were more prominent in memory Treg cells in unstimulated and stimulated conditions (Figure 4C–F and Figure S3D,E).

3.5 | Decreased CTLA-4 protein expression level of CD4^{+} FOXP3^{+} memory Treg cells in LRBA deficiency and CTLA-4 insufficiency

Unstimulated nTcons are devoid of CTLA-4 protein, thus can be used as a negative control during the evaluation of CTLA-4 expression.[22–24] We used the CTLA-4 level of nTcons as a divider to assess the upregulation of intracellular CTLA-4 expression in CD4^{+} FOXP3^{+} memory Treg cells after 16 h T-cell stimulation and compared the results for each disease with age-matched healthy controls (Figure 5A,B). We found that at baseline the relative CTLA-4 expression levels were lower in *LRBA*$^{-/-}$ (1.8 ± 0.5) and *CTLA4*$^{+/-}$ (2.2 ± 0.9) patients compared to healthy controls (2.9 ± 0.6, Figure 5B). When each group was compared to itself before and after the stimulation, CTLA-4 protein expression was found to be increased by stimulation in both diseases and healthy controls ($p = 0.0015$, $p = 0.0020$, $p < 0.0001$, respectively). Although CTLA-4 expression was increased in both diseases, it was still reduced compared to the healthy controls, and there was no difference between both diseases (Figure 5B).

3.6 | Induction of CTLA-4 protein expression was more apparent in LRBA deficiency compared to CTLA-4 insufficiency

We evaluated fold change increase of CTLA-4 in memory CD4^{+} FOXP3^{+} Treg cells (Figure 5C,D). After brief stimulation, the most prominent fold increase of CTLA-4 level was observed in age-matched healthy controls (3.5 ± 1.6) and *LRBA*$^{-/-}$ patients (3.4 ± 1.7), while *CTLA4*$^{+/-}$ patients demonstrated less induction (1.7 ± 0.4) compared to *LRBA*$^{-/-}$ patients, controls, and other IEI patients ($n = 8$) who were evaluated for CTLA-4 expression due to the recurrent infections and autoimmunity with normal genetic analysis for *LRBA* and *CTLA4* genes. The CTLA-4 levels decreased only in *LRBA*$^{-/-}$ and *CTLA4*$^{+/-}$ patients, demonstrating a specific defect regarding to these disorders (Figure 5E and Figure S3F).

We used anti-CTLA-4 antibody clone BNI3 to determine the level of CTLA-4 across the different mutations. In general, severe mutations such as stop gain or frameshift can lead to more profound CTLA-4 insufficiency; however, some missense mutations may result in normal protein expression, posing an underdiagnosis

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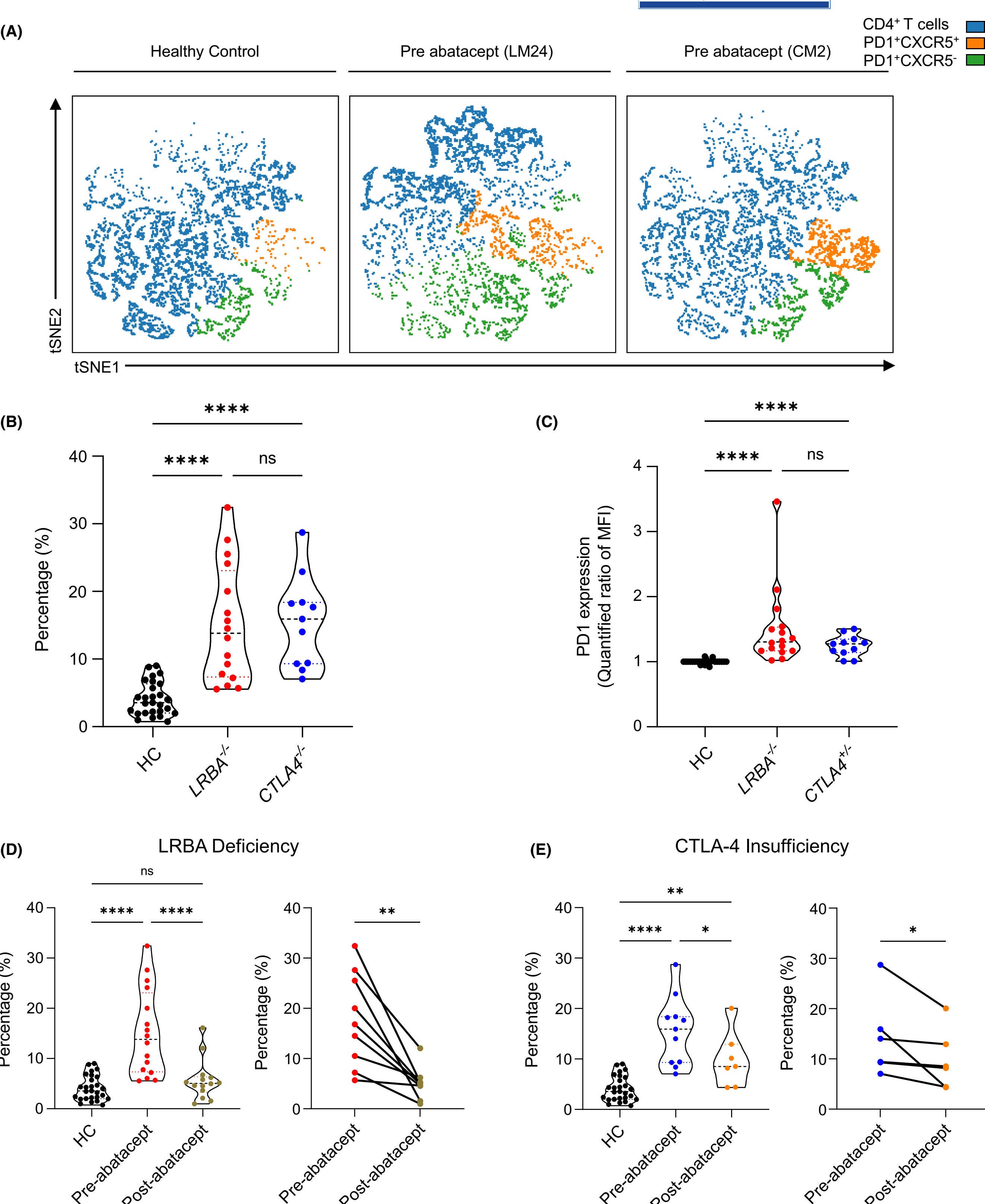

FIGURE 3 Increased activated cT_{FH} cells at baseline normalizing after abatacept in both disorders. (A) Representative t-distributed stochastic neighbor embedding (t-SNE) visualization of cT_{FH} of patients and age-matched healthy control with indicated colors. (B) The percentages of cT_{FH} in patients before abatacept. (C) PD-1 expression on patients' cT_{FH} cells before abatacept quantified to healthy controls. (D) The percentages of cT_{FH} in LRBA-deficient patients pre- and post-abatacept. (E) The percentages of cT_{FH} in CTLA-4-deficient patients pre- and post-abatacept. cT_{FH}, circulating T follicular helper cell; PD1, Programmed cell death protein 1; $LRBA^{-/-}$, LRBA deficiency; $CTLA4^{+/-}$, CTLA-4 insufficiency; ns, non-significant; MFI, mean fluorescence intensity. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, * $p < 0.05$, 1-way ANOVA with Tukey post-test and paired 2-tailed t test

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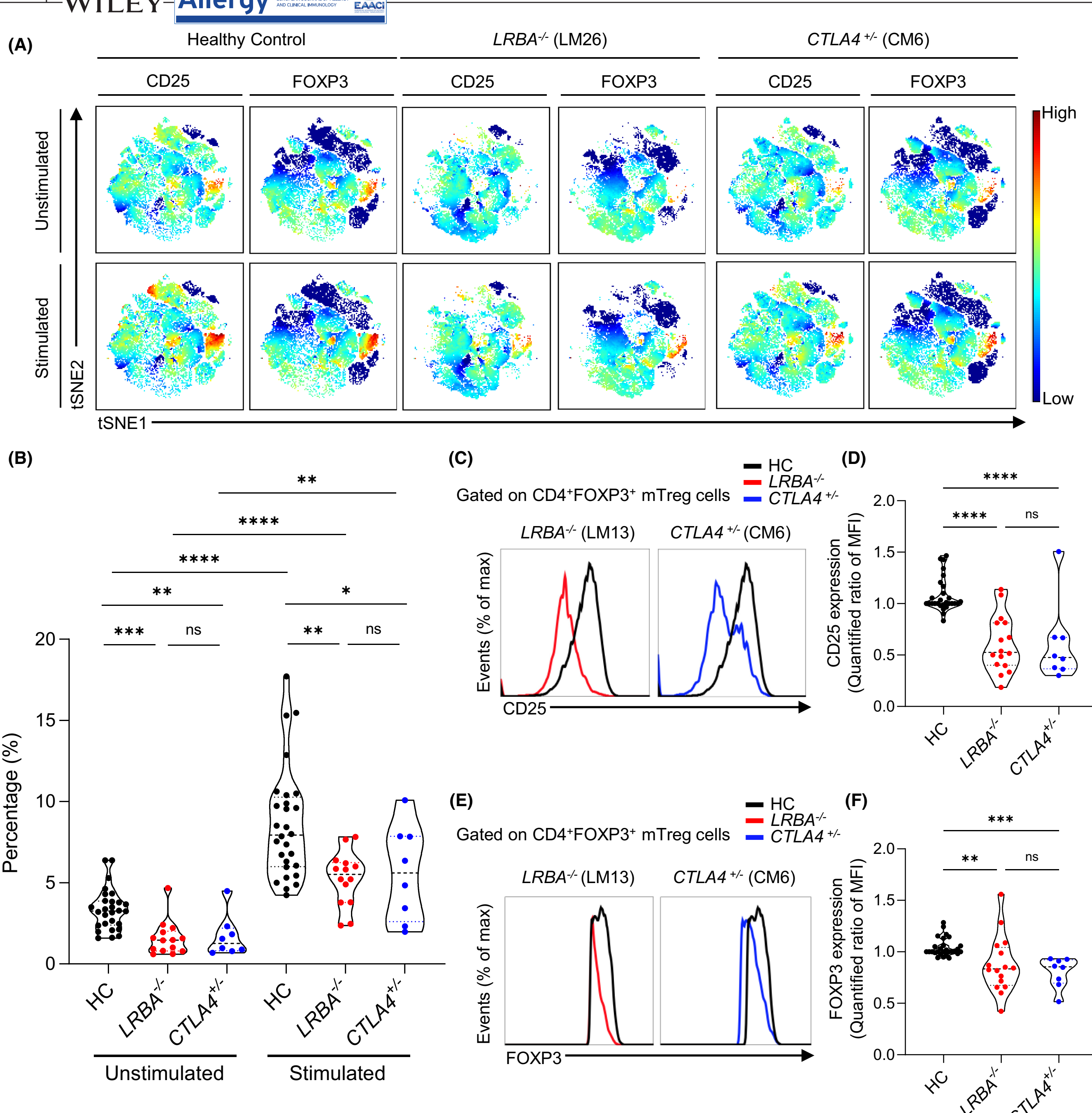

FIGURE 4 Reduced Treg cells in LRBA-deficient and CTLA-4-insufficient patients. (A) Representative t-distributed stochastic neighbor embedding (t-SNE) z-channel visualization of CD4^{+} CD25^{+} FOXP3^{+} Treg cells of patients and healthy controls. High expression indicates the levels of CD25 and FOXP3 within CD3^{+} CD4^{+} T cells. Treg cells staining is compared between unstimulated (top panels) or T cells stimulated (bottom panels) with anti-CD2, anti-CD3, and anti-CD28 beads for 16 h. (B) The percentages of CD4^{+} CD25^{+} FOXP3^{+} Treg cells in *LRBA*$^{-/-}$ ($n = 14$), *CTLA4*$^{+/-}$ ($n = 8$) and age-matched healthy controls ($n = 28$). (C) Representative histograms of CD25 expression in CD4^{+} FOXP3^{+} memory Treg cells. (D) The normalized mean fluorescence intensity of CD25 in *LRBA*$^{-/-}$ ($n = 16$), *CTLA4*$^{+/-}$ ($n = 8$) and healthy controls ($n = 28$) in unstimulated condition. (E) Representative histograms of FOXP3 expression in CD4^{+} FOXP3^{+} memory Treg cells. (F) The normalized mean fluorescence intensity of FOXP3 in *LRBA*$^{-/-}$ ($n = 16$), *CTLA4*$^{+/-}$ ($n = 8$) and age-matched healthy controls ($n = 28$) in unstimulated condition. *LRBA*$^{-/-}$, LRBA deficiency; *CTLA4*$^{+/-}$, CTLA-4 insufficiency; ns, non-significant; MFI, mean fluorescence intensity. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, 1-way ANOVA with Tukey post-test and paired 2-tailed t test

of CTLA-4 insufficiency.[15,22] To determine the effect of the mutation on CTLA-4 protein levels, we used a quantified MFI ratio generated by dividing the patients' CTLA-4 levels by the healthy controls' levels. There were no differences in CTLA-4 levels in unstimulated condition between patients with the various mutation types, demonstrating the persistent decreased level of CTLA-4 expression in all patients, even with missense mutations (Figure S4).

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Finally, we analyzed the CTLA-4-fold change cutoff values by receiver operating characteristic analysis to determine an appropriate specificity and sensitivity that differentiates *CTLA4*$^{+/-}$ from *LRBA*$^{-/-}$ patients. Our results revealed higher sensitivity (87.5%) and specificity (90%) by using a cutoff value less than 1.96 for the CTLA-4-fold change increase with an area under curve of 0.94 and a 95% CI (0.83–1) ($p = 0.001$, Figure 5F). This cutoff value was able to identify all *CTLA4*$^{+/-}$ patients.

Collectively, our clinical and laboratory results show successful stepwise clinical and laboratory approaches to distinguish *LRBA*$^{-/-}$ and *CTLA4*$^{+/-}$ patients. Based on our results, we developed an algorithm that can guide the genetic analysis of patients suspected to have LRBA deficiency or CTLA-4 insufficiency (Figure 6).

4 | DISCUSSION

In this study, we provided a head-to-head comparative evaluation of clinical and immunological features of LRBA deficiency and CTLA-4 insufficiency. Patients presented with various shared phenotypes such as infection susceptibility, ID, and LP. LRBA-deficient patients showed an early-onset, more severe clinical course than CTLA-4 insufficiency. In LRBA deficiency, the OS probability was not different between transplanted versus patients on long-term immunosuppressants, but was lower compared to the CTLA-4 insufficiency. The T-cell subsets showed more deviation to memory cells in CTLA-4-insufficient patients, accompanied by low percentages of Treg and dysregulated cT_{FH} cells in both diseases, and cumulative numbers of autoimmunities positively correlated with cT_{FH} frequencies. Baseline CTLA-4 expression was significantly diminished in both diseases compared to healthy controls, but partially recovered after brief T-cell stimulation, a pattern which was less prominent in CTLA-4 patients.

Both diseases are characterized by CVID and ALPS-like phenotypes, presenting with a predominance of early-onset and forms of autoimmunities such as antibody-mediated cytopenias, enteropathy, type 1 diabetes, and other endocrinopathies.[14,15,25–29] Hypogammaglobulinemia, organomegaly, and unusual lymphocytic infiltration of many organs including lung and brain can be observed during the course of these diseases.[6,30] Hyperproliferative activity of lymphoid cells, increased inflammatory responses, and impaired control of oncogenic viruses, mainly EBV, also increase the susceptibility to malignant transformation especially favoring the occurrence of lymphomas and gastric cancer.[11,31,32] In general, LRBA-deficient patients are more prone to lung infections and autoimmune enteropathy associated with FTT.[33] Although both disorders impact the same immune pathway, why LRBA deficiency commonly presents earlier with life-threatining infections compared to CTLA-4 insufficiency is precisely unknown. One of the potential factors that explains the differences between disorders is the variability of disease penetrance, usually reported in CTLA-4 insufficiency, which is nearly complete in LRBA deficiency.[15,33] Furthermore, asymptomatic patients of our cohort were indistinguishable from those with overt disease presentation in terms of level of CTLA-4 expression, percentage of Treg, and upregulation of CTLA-4 upon stimulation, demonstrating the possibility of other drivers like genetic and epigenetic modifiers or environmental changes.

Our study revealed similar OS between transplanted and non-transplanted LRBA-deficient patients. It is worth noting that after successful transplantation, the patients stopped their medications, while the non-transplanted patients continued to receive IgRT and/or immunosuppressants to control disease activity. Our study clearly demonstrates the importance of early diagnosis, as the disease burden reversely influences the effectiveness of both drug therapies and HSCT.[13,17] We also demonstrated that abatacept can be successfully applied to patients for long-term period without major side effects and stabilizes the patients to offer better outcome for the following transplantations. From the therapeutic perspective, special evaluation should be performed for each patient, but initially considering early implementation of abatacept with close monitoring of effectiveness would be the best option for favorable outcome. In case of unresponsiveness to targeted therapy, patients should be evaluated timely for HSCT.

We found that LRBA-deficient and CTLA-4-insufficient patients exhibited lower $CD3^+$, $CD4^+$, and $CD8^+$ T cells, with the majority of them having an inverted naive to memory phenotype. These abnormalities were previously explained by the hyperproliferative responses with increased apoptosis due to loss of CTLA-4 function.[2,6,13] We also observed increased double-negative T and reduced recent thymic emigrants and NK cells in both diseases, which could contribute to the vulnerability to viral and fungal infections.[6,12,13] Interestingly, we detected a more severe immunological phenotype in CTLA-4 insufficiency, which was characterized by decreased naive $CD4^+$ and central memory T cells with increased effector memory $CD4^+$ T cells. It would be interesting to speculate whether the higher proportion of effector memory $CD4^+$ T cells in CTLA-4 insufficiency versus LRBA deficiency is due to the lower upregulation of CTLA-4 protein levels during activation.

Classically LRBA deficiency and CTLA-4 insufficiency have been categorized as Tregopathies, characterized by loss of Treg cell function, which is required for normal immune surveillance and tolerance.[34,35] Interestingly, a variable frequency of Tregs has been reported in different disease cohorts, ranging from normal to reduced or increased levels.[3,9,11,22] Factors such as patients' clinical status, CD4 number, immunosuppressive treatments, and time of analysis after blood draw might influence the Treg numbers in tested patients. Importantly, the imbalanced CTLA-4 and CD28 pathways result in greater availability of CD80/CD86 to bind CD28 molecule, which can promote expansion of Treg cells.[3] On the contrary, activated conventional T cells can transiently express FOXP3, which can influence the accuracy of measuring the real Treg frequencies by flow cytometry.[36] Barzaghi et al. consistently showed a quantitative demethylation defect in the *FOXP3* locus of patients with IPEX-like syndrome, even in patients with normal Treg numbers.[37] Therefore, measuring the methylation status of $FOXP3^+$ cells would help discriminate between real Treg and activated conventional T cells, as

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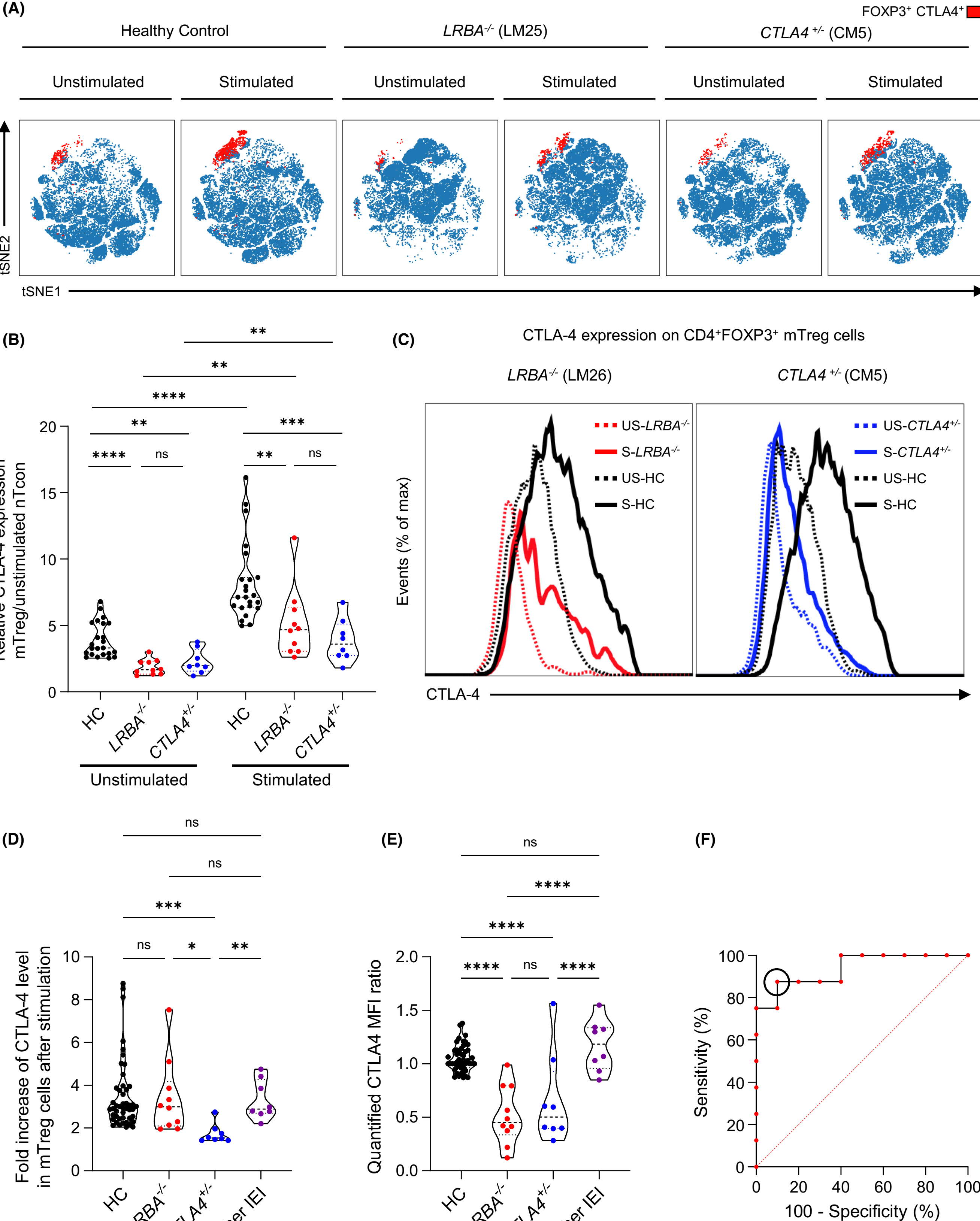

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FIGURE 5 LRBA deficiency demonstrates low CTLA-4 expression, recovered after stimulation. (A) Representative t-distributed stochastic neighbor embedding (t-SNE) visualization of CTLA-4 in memory CD4$^+$ FOXP3$^+$ Treg cells in patients and healthy controls with indicated colors. CTLA-4 expression is compared between unstimulated (top panels) or T cells stimulated (bottom panels) with anti-CD2, anti-CD3, and anti-CD28 beads for 16 h. (B) Relative CTLA-4 mean fluorescence intensity in mTregs of *LRBA*$^{-/-}$ ($n = 10$), *CTLA4*$^{+/-}$ ($n = 8$), and healthy controls ($n = 23$) normalized to the MFI of nTcons. (C) Representative histograms of CTLA-4 expression in CD4$^+$ FOXP3$^+$ memory Treg cells before and after 16 h stimulation with anti-CD2, anti-CD3, and anti-CD28 beads. (D) The fold increase of CTLA-4 in mTreg cells after stimulation in *LRBA*$^{-/-}$ ($n = 10$), *CTLA4*$^{+/-}$ ($n = 8$), other IEI ($n = 14$), and age-matched healthy controls ($n = 23$). (E) The normalized unstimulated mean fluorescence intensity of CTLA4 in *LRBA*$^{-/-}$ ($n = 10$), *CTLA4*$^{+/-}$ ($n = 8$), other IEI ($n = 8$), and healthy controls ($n = 23$). (F) Receiver-operating characteristic curve analysis shows the higher sensitivity and specificity (black circle) of flow cytometric analysis in distinguishing CTLA-4 insufficiency from LRBA deficiency. Area under the curve was 0.94 with a 95% CI (0.83–1). *LRBA*$^{-/-}$, LRBA deficiency; *CTLA4*$^{+/-}$, CTLA-4 insufficiency; ns, non-significant; MFI, mean fluorescence intensity; US, unstimulated; S, stimulated. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, 1-way ANOVA with Tukey post-test, Kruskal–Wallis with Dunn's post-test and paired 2-tailed *t* test

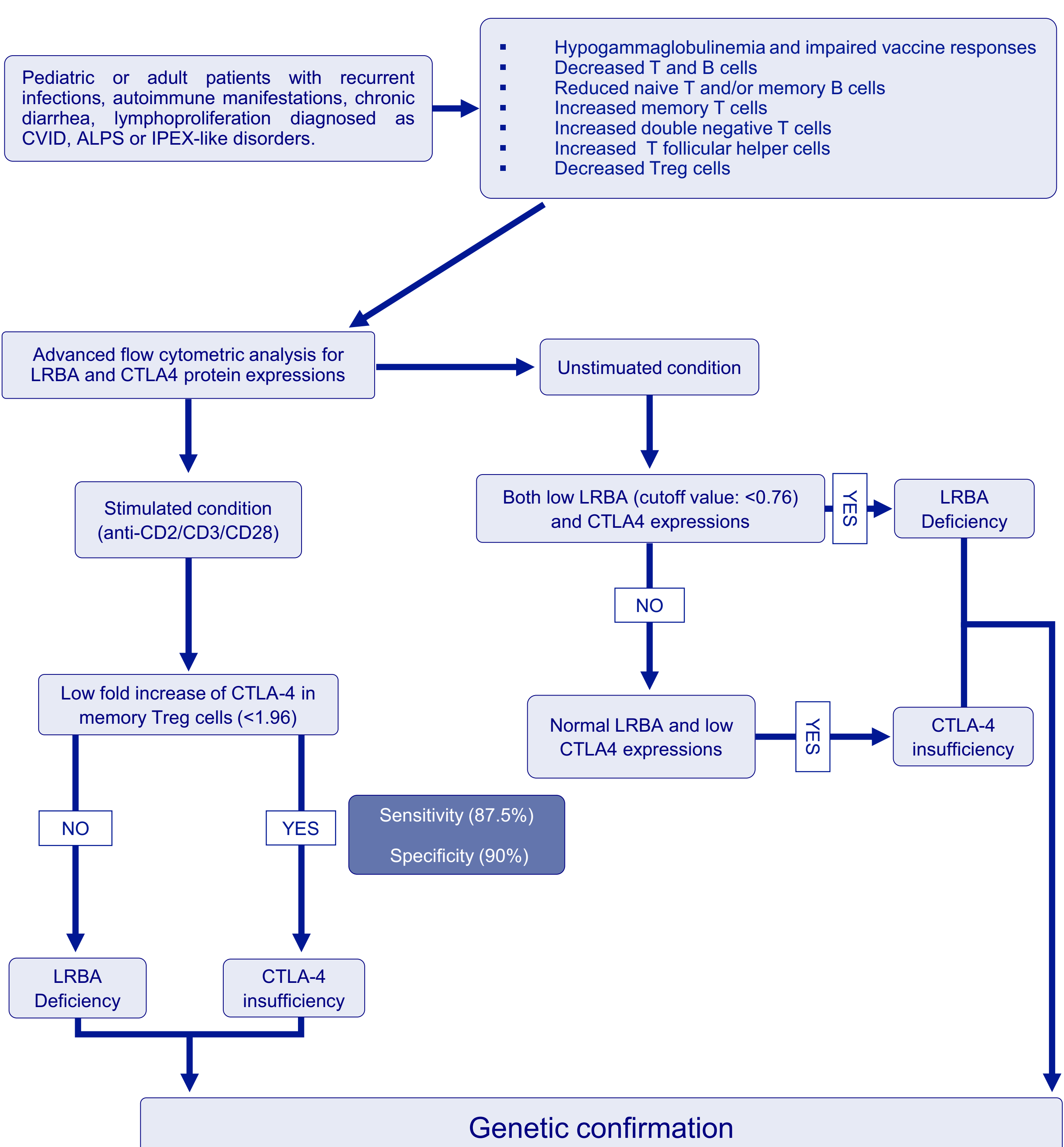

FIGURE 6 Flowchart for diagnosis of LRBA deficiency and CTLA-4 insufficiency via clinical and flow cytometric features

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the latter would display greater methylation of the *FOXP3* locus. Furthermore, it can be conceivable that measurement of the methylation status of the *FOXP3* locus may clarify the heterogeneity of the clinical findings of these patients, especially in CTLA-4 insufficiency. Further investigations are warranted to explore the Treg abnormalities in both diseases.

Previously, Hou et al[22] described a diagnostic assay methodology based on CTLA-4 intracellular expression and discovered that LRBA-deficient patients, similar to healthy controls, transiently recovered their diminished CTLA-4 expression on Tregs after short stimulation, confirming that there is no defect in CTLA-4 production, although could not prevent enhanced degradation due to the lack of LRBA. By using this approach, we identified that LRBA-deficient patients cannot fully recover to normal CTLA-4 levels, and after stimulation, they still displayed less CTLA-4 expression than controls. Nevertheless, our results demonstrated evidence of increased CTLA-4-fold change after stimulation, distinguishing LRBA deficiency from CTLA-4 insufficiency with high sensitivity and specificity. The variability between studies may relate to the technical variations across different laboratories, indicating that every center should set its own reference values.

Of note, some CTLA-4-insufficient patients have mutations causing normal protein expression but disturbed function, as demonstrated in previous studies of CTLA-4 missense mutations evaluated by transendocytosis or ligand uptake assays.[3,8,22] Therefore, in all suspected patients, mutation analysis should be offered for definitive diagnosis.

In conclusion, the presented large cohort provided detailed clinical and laboratory comparisons of the defects related to the CTLA-4 pathway. A step-by-step diagnostic algorithm can be helpful to discriminate between the diseases, thus facilitating the use of targeted sequencing to provide rapid diagnosis and treatment.

AUTHOR CONTRIBUTIONS

S.B. conceptualized and supervised the study. M.C.C., B.A., G.A., D.B., A.B., F.B., and Y.D.K. performed the experiments. S.B.E., R.B., I.S.K., N.K, A.K., G.H., S.I.K.D, S.O., S.C., D.K., N.E.K., A.S.S., M.C., E.O.Y., Z.T., D.U.A., C.A., F.C., H.C., N.G., F.G., A.M., S.N.G., N.K., S.K., I.R., S.S.K., A.Y., E.K.A, A.O., and S.B. provided patient care, and collected samples and clinical data. S.B., M.C.C, B.A., and I.S.K. wrote the paper. B.L. edited the manuscript. All authors reviewed and approved the final version of the manuscript.

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None.

CONFLICT OF INTEREST

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