



FULL LENGTH ARTICLE

Abatacept is effective in Chinese patients with LRBA and CTLA4 deficiency

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Abstract CTLA4 deficiency and LRBA deficiency are a group disorders of immune dysregulation that affect CTLA4 pathway. The patients mainly present with autoimmunity, antibody deficiency and recurrent infections. Here we reported three Chinese patients with LRBA and CTLA4 mutations. They all presented with chronic diarrhea, hypokalemia, organomegaly, recurrent infections, and hypogammaglobulinemia. Reduced Treg cells and increased percentage of circulating follicular helper T (cTfh) cells were revealed in these patients. Although steroid and immunoglobulin therapy were given, the enteropathy was persistent. Therefore, abatacept treatment was provided to these patients. They showed a marked improvement of enteropathy and gastrointestinal endoscopy showed alleviated inflammatory lesion and follicular hyperplasia. Furthermore, the frequency of cTfh cells was reduced after abatacept therapy. Taken together, targeted therapy with abatacept is a promising treatment modality for patients with LRBA and CTLA4 deficiency. The findings also suggest that the frequency of cTfh cells could serve as a marker for tracking disease activity and the response to abatacept therapy.

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Introduction

Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is an inhibitory checkpoint protein, expressed on activated T cells and Treg cell subsets.¹ CTLA4 inhibits immune responses by competing with the costimulatory molecule CD28 for the ligands CD80 and CD86, or by removing these ligands from antigen-presenting cells by transendocytosis, thus playing a critical role in suppressing autoimmunity and maintaining immune homeostasis.² Heterozygous loss-of-function mutations in *CTLA4* in human cause an autosomal-dominant immune dysregulation syndrome.^{3–5} As an important control point for the lysosomal turnover of CTLA4 protein in T lymphocytes, LPS-responsive beige-like anchor (LRBA) helps maintain intracellular stores of CTLA4.⁶ Biallelic mutations in *LRBA* also result in a similar disease phenotype.^{3,7} Patients with LRBA and CTLA4 deficiency present with hypogammaglobulinemia, organomegaly, autoimmune disorders, chronic diarrhea, and recurrent infections alone or in combination.^{8–10} Regular intravenous immunoglobulin therapy (IVIG) is commonly used after the diagnosis of hypogammaglobulinemia and could effectively reduce the frequency of bacterial infections. For treatment of autoimmune diseases, different agents including corticosteroids, immunosuppressive drugs, rituximab and infliximab have been applied but most patients failed to get sustained improvement.^{6,10,11} Recently, abatacept has been suggested as a promising therapy for improving immune dysregulatory symptoms in patients.^{6,12}

In this study, we reported the clinical features and treatments of two patients with LRBA deficiency and one with CTLA4 deficiency. The long-term efficacy of abatacept therapy was demonstrated for controlling enteropathy.

Materials and methods

Patients

Three patients from non-consanguineous families were enrolled in this study. The clinical manifestations and detailed treatments including steroids, IVIG, immunosuppressants as well as abatacept were collected for each patient. This study was approved by the Medical Ethics Committee of the Children's Hospital of Chongqing Medical University (030/2013). A written informed consent was obtained from all parents.

DNA and RNA extraction

Genomic DNA (gDNA) was extracted from 200 μ l whole blood using the QIAamp DNA Mini Kit (Qiagen GmbH, Germany). Total RNA was isolated from 200 μ l whole blood using the Total RNA Miniprep Kit (Axygen, China) and

subjected to reverse transcription-polymerase chain reaction (RT-PCR) using the EvoScript Universal cDNA Master (Roche Diagnostics GmbH, Germany) according to the manufacturers' instructions.

Genetic analysis

Whole-exome sequencing (WES) was performed on genomic DNA of three patients. Mutations in the *LRBA* and *CTLA4* gene were verified by Sanger sequencing for patients and their parents. The maternal family members of patient 3 were also subjected to Sanger sequencing.

Antibodies and flow cytometry

Antibodies to the following makers were used for staining: CD3 (HIT3A), CD4 (RPA-T4), CD45RA (HI100), CD25 (BC96), CTLA4 (BNI3), CD45RO (UCHL1), CXCR5 (RF8B2), PD-1 (EH12.2H7), FOXP3 (PCH101) and the appropriate isotype controls. Whole blood was incubated with mAbs against surface markers for 30 min on ice. Freshly isolated PBMCs were fixed and permeabilized by using eBioscience Fixation/Permeabilization (eBioscience, San Diego, CA), then intracellular staining with anti-FOXP3 or anti-CTLA4 antibody was performed. Data were collected with a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Statistical analysis

Data were analyzed using an unpaired two-tailed Student *t* test. All statistical analyses were conducted in GraphPad Prism 7 software (GraphPad Software, Inc., San Diego, CA). $P \leq 0.05$ was considered to indicate a significant difference.

Results

Clinical manifestations of the patients

In family A the index case (patient 1) presented to us at 12 years of age with recurrent non-infectious diarrhea and hypokalemia. She also had chronic kidney disease, splenomegaly, lymphadenopathy, bronchiectasis and hypogammaglobulinemia. Colonoscopy revealed mucosal erosion and superficial ulcer of sigmoid colon and rectal. In family B the index case (patient 2) is a 15-year-old boy, he presented with chronic diarrhea when he was 4 years old. Other clinical features included hepatosplenomegaly, lymphadenopathy and autoimmune diseases including autoimmune hemolytic anemia and autoimmune thrombocytopenia. In family C, patient 3 is a 13-year-old boy who manifested with chronic diarrhea, persistent hypokalemia

and dysfunction of liver at 10 years of age. Hepatosplenomegaly and multiple lymphadenopathy were noted. Gastrointestinal endoscopy revealed duodenal, ilea and colonic inflammatory follicular hyperplasia. He also suffered from recurrent infections including pneumonia, bronchitis, cellulitis, otitis media, varicella and fungal infections.

Genetic analysis revealed *LRBA* and *CTLA4* mutations in patients

Based on the clinical symptoms and laboratory results, immunodeficiency was considered for the three patients and whole-exome sequencing was performed. Compound heterozygous mutations in *LRBA* were found in patient 1 (c.1897C > T, p.R633X; c.5519-1G > T) (Fig. 1A and B). TA cloning of amplicons from patient 1 indicated this splicing mutation resulted in the deletion of exon 34. Patient 2 also had a splicing mutation in *LRBA* (c.8349+1G > A), leading to the deletion of exon 56 (Fig. 1C). Furthermore, analysis of copy number variations in patient 2 identified one deletion mutation which led to the absence from exon 22 to exon 36 of *LRBA* (Fig. 1D). Patient 3 had a heterozygous insertion mutation (c.211–212 ins TGACAGTGCTTCGGCAGGC) in exon 2 of *CTLA4* which leads to a pre-stop codon (p.Q80DfsX4) (Fig. 1E). His mother and grandfather were asymptomatic carriers (Fig. 1A).

Patients had reduced Treg cells and *CTLA4* expression

All of the patients showed gastrointestinal autoimmunity and patient 2 also had autoimmune hematologic disorders. Considering the importance role of Treg cells in suppressing autoimmunity, we evaluated the percentage of Treg cells using flow cytometry. It revealed a profound decrease in the frequency of CD25⁺FOXP3⁺ Treg cells in all patients (control subjects: 8.5% ± 0.8% vs patients: 4.6% ± 0.7%; Fig. 2A and B). The mother of patient 3 also showed reduced Treg cells (Fig. 2A). His grandfather was not available for analysis. We further detected the expression of Treg function-associated molecules, the results showed reduced *CTLA4* expression in all patients and the mother of patient 3 (Fig. 2C).

Patients had increased CXCR5⁺PD1⁺ circulating follicular helper T cells

Given that circulating follicular helper T (cTfh) cells play an important role in disease-related autoimmunity, we next examined the frequency of cTfh cells before abatacept therapy by flow cytometry. Compared with healthy controls ($n = 10$), all patients showed significantly increased percentage of CXCR5⁺PD1⁺ cTfh cells in CD4⁺ T cells (control subjects: 4.6% ± 0.3% vs. patients: 23.4% ± 5.0%; Fig. 3A and B).

Treatment and outcome

All patients received regular intravenous immunoglobulin replacement because of hypogammaglobulinemia and recurrent infections. During follow-up, serum immunoglobulin detection revealed normalized IgG levels. The frequency of respiratory infections in patients also reduced. Systemic steroids were used to control autoimmune features in all patients after diagnosis of *LRBA* and *CTLA4* deficiency. Patient 3 was also treated with mycophenolate mofetil as steroids alone failed to alleviate the symptoms of diarrhea. However, the treatment of steroids in combination with immunosuppressive drugs did not improve the patients' condition. The side effects including concentric obesity, osteoporosis, opportunistic infections and glucocorticoid induced oculopathy were revealed in two patients. Considering the autoimmune disorders in patients may be associated with reduced *CTLA4* expression, target therapy with abatacept was given to patients twice every 3 weeks at dose of 125 mg subcutaneously. The clinical and immunologic features were evaluated every three months. Patient 1 respond well to abatacept for that she had no diarrhea since the third month after abatacept. Consistent with the clinical improvement, follow up gastrointestinal endoscopy of patient 1 performed at ninth month showed alleviated inflammatory lesion in colon (Fig. 4A). The frequency of diarrhea also reduced in patient 3 and gastrointestinal endoscopy performed at sixth month revealed the duodenal, ilea and colonic follicular hyperplasia was obviously reduced (Fig. 4A). Follow up immunologic studies revealed decreased percentage of cTfh cells in the two patients (Fig. 4B). Patient 1 stopped abatacept therapy after ten months because she could not afford the drug. Four months later, she showed diarrhea again and flow cytometric analysis revealed increased frequency of cTfh cells (Fig. 4B). Patient 2 started abatacept therapy recently, the frequency of diarrhea reduced but more time is needed to evaluate the long-term efficacy.

Discussion

Patients with *LRBA* and *CTLA4* deficiency present with a broad spectrum of clinical manifestations including hypogammaglobulinemia, recurrent infections, lymphadenopathy, splenomegaly, autoantibody-mediated cytopenia, organ-specific autoimmunity, and lymphocytic infiltration of nonlymphoid organs.^{8,10} In our study, the common clinical features of the three patients were chronic diarrhea, recurrent infections, hypogammaglobulinemia and organomegaly. Chronic diarrhea was the most severe symptom and caused a series of complications including hypokalemia, malnutrition and growth retardation. Patient 2 also suffered from autoimmune hemolytic anemia and autoimmune thrombocytopenia. While carrying the same *CTLA4* mutation, the mother and grandfather of patient 3 were asymptomatic. The blood sample of his mother was available for analyzing the functional change. Decreased *CTLA4* expression and Treg cells were detected in his mother. Recent studies reported the penetrance in human *CTLA4* insufficiency was 67.6%–71.2%, and the factors responsible for the incomplete penetrance may include gender,

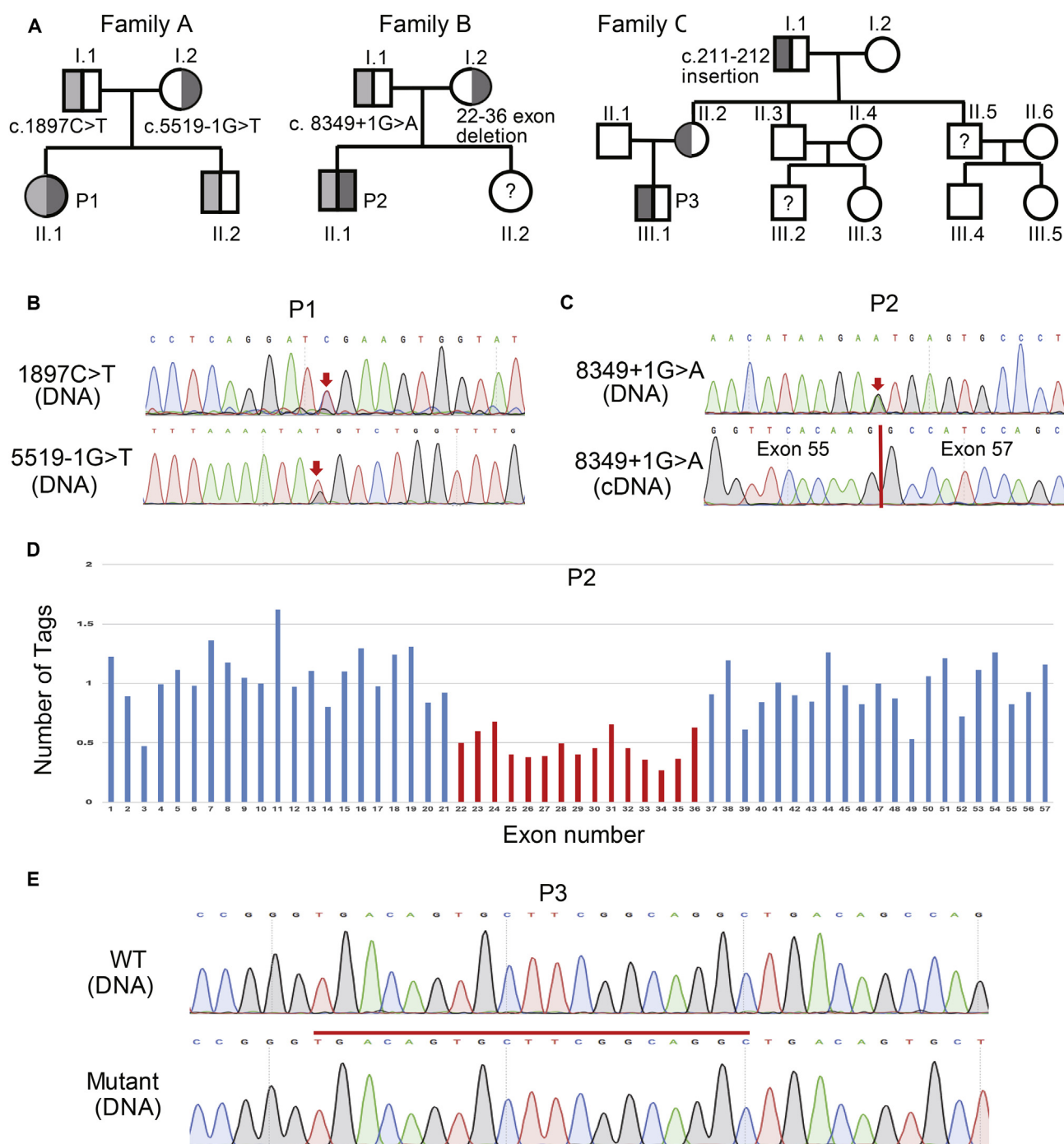


Figure 1 Pedigrees and mutational analysis of the LRBA-deficient and CTLA4-deficient patients. **(A)** Pedigree of family A, B and C. Generations are designated by Roman numerals (I–II), and subjects are designated by Arabic numerals. Mutations are indicated below subjects. Proband in the respective families are indicated as P1 to P3. Squares, Male subjects; circles, female subjects. Half-solid symbols, heterozygous carriers. Circles marked with two colors, patients with compound heterozygous mutations. **(B)** Sanger sequencing of compound heterozygous mutations in patient 1. Red arrows indicate the location of mutations. **(C)** Sanger sequencing of DNA and cDNA from patient 2 proved the c.8349+1G > A mutation resulted in the deletion of exon 56. **(D)** Analysis of copy number variations in patient 2 revealed a deletion mutation from exon 22 to exon 36. **(E)** Sanger sequencing identified an insertion mutation of TGACAGTGCTTCGGCAGGC in patient 3.

microbial pathogens, or the variation of internal threshold of immune system.^{13–15} The search for definite modifying factors in CTLA4 insufficiency still continues. More affected and unaffected mutation carriers are needed to identify

possible factors that can influence the occurrence of clinical symptoms.

For patients with LRBA and CTLA4 deficiency, autoimmune complications including cytopenia and enteropathy

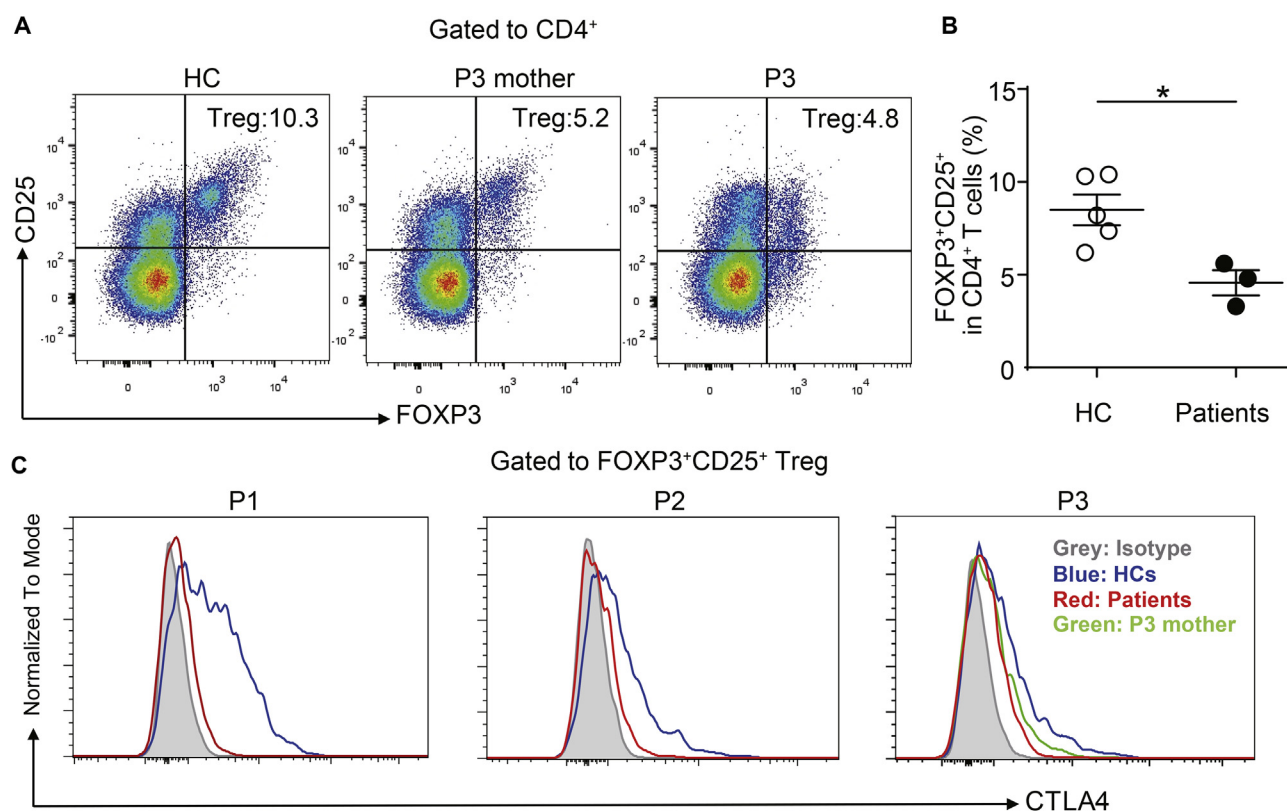


Figure 2 Patients with LRBA and CTLA4 deficiency showed reduced Treg cells and CTLA4 expression. (A) The frequency of FOXP3⁺CD25⁺ Treg cells in patient 3 and his mother (P3 mother) was presented. (B) Percentage of FOXP3⁺CD25⁺ Treg cells in CD4⁺ cells from patients ($n = 3$) versus control subjects ($n = 5$). (C) Compared with healthy controls, the CTLA4 expression of Treg cells in patients and the mother of patient 3 was reduced.

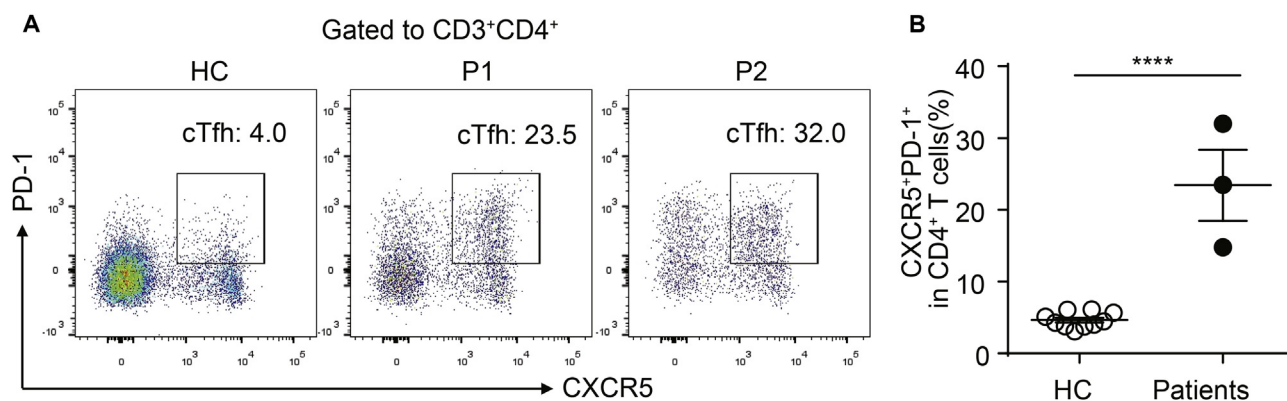


Figure 3 Patients with LRBA and CTLA4 deficiency showed high frequency of cTfh cells. (A) Flow cytometric analyses of CXCR5⁺PD-1⁺ cTfh cells in CD4⁺ T cells in patient 1 and 2 before treatment with abatacept. (B) Frequency of cTfh cells from patients ($n = 3$) versus control subjects ($n = 10$).

were the most life-threatening and treatment-resistant manifestations. To manage autoimmune and inflammatory diseases, most patients received systemic corticosteroids or in combination with immunosuppressive drugs including cyclosporine, mycophenolate mofetil, sirolimus, tacrolimus, sulfasalazine, or azathioprine.⁸ Abatacept is a recombinant fusion protein comprising the extracellular domain of human CTLA4 and a fragment of the Fc domain of human IgG1, which has been modified to prevent

complement fixation.¹⁶ Like CTLA4, abatacept competes with CD28 for CD80 and CD86 binding and thereby can be used to selectively modulate T-cell activation.¹⁷ Bernice Lo et al first reported patients with LRBA deficiency showed CTLA4 loss and immune dysregulation responsive to abatacept therapy.⁶ In our study, all patients received systemic steroids as a routine therapy to control autoimmune features, but the diarrhea was persistent and a series of side effects appeared. Therefore, targeted therapy with

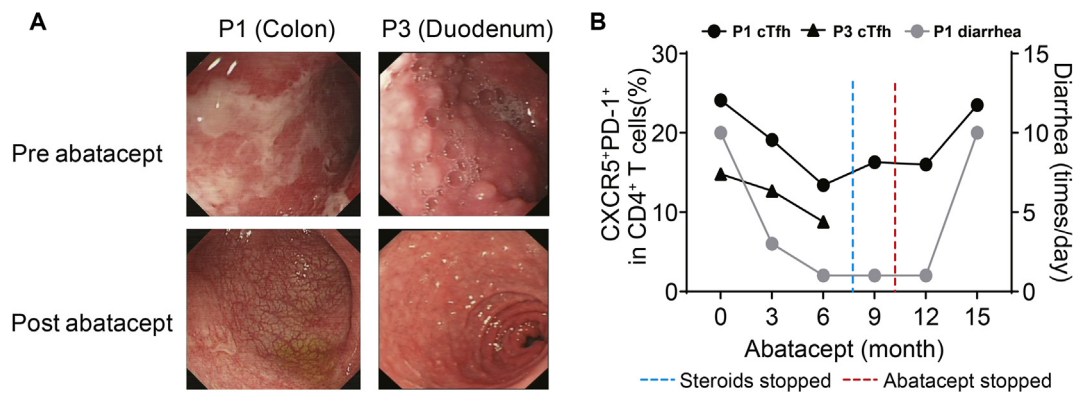


Figure 4 Patients with LRBA and CTLA4 deficiency respond well to abatacept therapy. **(A)** Gastrointestinal endoscopy before and after abatacept therapy. Left panel, patient 1; right panel, patient 3. **(B)** cTfh cell frequencies in patient 1 (black dots) and patient 3 (black triangles), as well as the times of diarrhea in patient 1 (grey dots) before and after abatacept therapy.

abatacept was initiated. Both patient 1 and patient 3 demonstrated significant improvement in clinical condition, as evidenced by the reduced diarrhea clinically and alleviated inflammatory lesion and follicular hyperplasia by gastrointestinal endoscopy. The lymphadenopathy and hepatosplenomegaly also showed remission. In addition, the dose of steroids gradually reduced and finally ceased during the period of abatacept treatment. Our findings were consistent with another report, where the patients with LRBA deficiency showed good response to abatacept treatment in terms of the remission of lymphoproliferation, chronic diarrhea and immune dysregulation.¹² Furthermore, we found that after stopping abatacept in patient 1, the diarrhea appeared again four months later, which, therefore, suggests long-term treatment with abatacept is important in controlling the disease activity.

Tfh cells are required for maintenance of the germinal center reaction, generation of high-affinity antibody, and long-lasting plasma and memory B cells.¹⁸ Associations between autoimmunity and overactive Tfh cells have been suggested in human systemic lupus erythematosus, rheumatoid arthritis, and several primary immunodeficiency diseases, including in STAT1 gain of function patients.^{19,20} The reduced CTLA4 expression on Treg cells was a key mechanism underlying the dysregulated Tfh cell response.^{21,22} Therefore, cTfh cells might play a critical role in disease-related autoimmunity.²¹ In this study, we showed that patients with LRBA and CTLA4 deficiency have decreased expression of CTLA4 while high frequency of cTfh cells compared with normal controls. After abatacept therapy, the percentage of cTfh in patient 1 and patient 3 gradually decreased during the follow-up, which was consistent with the alleviated clinical manifestations. Patient 1 showed non-infectious diarrhea again when stopped abatacept and flow cytometric analysis revealed increased frequency of cTfh cells at the same time. Thus monitoring cTfh cell in patients with LRBA and CTLA4 deficiency might be particularly useful in tracking disease activity.

Treg cells play an indispensable role in maintaining immunological tolerance to self-antigens and in suppressing excessive immune responses deleterious to the host.²³ Decreased frequency, aberrant phenotype, and decreased

suppressive function of Treg cells were reported in patient with LRBA deficiency.^{24,25} In this study, all patients with LRBA and CTLA4 deficiency showed decreased percentage of CD4⁺CD25⁺FOXP3⁺ Treg cells in CD4⁺T cells. These data together suggested that LRBA and CTLA4 may have an important role on the development of Treg cells. In addition, we demonstrated that the cTfh cells were increased in patients with LRBA and CTLA4 deficiency, while decreased after abatacept therapy. Previous study also found cTfh cell frequencies were inversely correlated with the CTLA4 expression on Treg cells.²¹ Thus CTLA4 may play an important role in controlling Tfh cell differentiation.

In conclusion, we reported the clinical manifestations, immunological features and treatments of two Chinese patients with LRBA deficiency and one with CTLA4 deficiency. Targeted therapy with abatacept is a promising treatment modality for these patients, even if they are refractory to conventional steroid and immunosuppressive drugs. In addition, the frequency of cTfh cells could serve as a sensitive marker for tracking disease activity and the response to abatacept therapy.

Conflict of Interests

The authors declare that they have no conflicts of interest.

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