European Review for Medical and Pharmacological Sciences 2020; 24: 4451-4460

The effect of Casitas b-lineage lymphoma b on regulating T follicular helper in lupus nephritis

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Abstract. – OBJECTIVE: The purpose of this study was to investigate the effect of Casitas b-lineage lymphoma b (Cblb) on the regulation of T follicular helper (Tfh) in the development of lupus nephritis.

MATERIALS AND METHODS: The Tfh (CD4+CXCR5+PD-1+) cells in peripheral blood were analyzed by flow cytometry. Forty mice were divided into 4 groups (10/group), WT, lpr, Cblb$^{-/-}$ and lpr.Cblb$^{-/-}$. Urine protein, serum creatinine, blood urea nitrogen (BUN), dsDNA, and antinuclear antibody (ANA) titer of mice were monitored once every four weeks. Peripheral blood mononuclear cells (PBMCs) from mice were collected to assess circulating Tfh. The expressions of Cblb in Tfh cells were regulated by transfecting siRNA and overexpression plasmid approach *in vitro*.

RESULTS: The patients with lupus nephritis (LN) had abnormal renal clinical manifestations compared with healthy volunteers. The peripheral Tfh cells were increased and the expression of Cblb were downregulated in patients with LN ($p<0.05$). Both lpr mice and lpr.Cblb$^{-/-}$ mice had LN symptoms. LN symptoms were more serious in lpr.Cblb$^{-/-}$ mice compared with that in lpr mice ($p<0.05$). The number of Tfh cells in peripheral blood from lpr.Cblb$^{-/-}$ mice was significantly higher than that from lpr mice ($p<0.05$). Overexpression of Cblb in Tfh cells led to reduction of IgG expression, while the knockdown of Cblb in Tfh cells was accompanied by increased expression of immunoglobulin (IgG) ($p<0.05$).

CONCLUSIONS: Cblb showed a negative regulatory effect on Tfh. The deletion of Cblb may be a key factor in progression of renal injury.

Key Words:
Lymphoma b, T follicular helper, PBMCs, Lupus nephritis.

Introduction

Lupus Nephritis (LN) is the most common and serious complication of systemic lupus erythematosus (SLE). End-stage nephropathy induced by LN is one of the leading causes of death in SLE. Therefore, understanding the pathogenesis of LN is of great importance. LN is mediated by immune complex and has a complex pathogenesis. It is currently believed that the involvement of helper T cells in the activation of B cells is an important part in the development of lupus nephritis[1]. In recent years, both *in vivo* and *in vitro* researches have confirmed the excessive activation of endogenous B cells in lpr mice. Patients with LN were associated with the production of autoantibodies, such as anti-dsDNA antibodies.

The universality or selectivity loss of the tolerance of helper T cells could result in the transformation of helper T cells into pathogenic T cells. These morbidly T cells are the main reason for the activation of polyclonal B cells[2,3]. T follicular helper (Tfh) cells are a subset of CD4+ T cells which localize in the germinal centres (GCs) of secondary lymphoid organs[4]. These specialized

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cells are crucial for the formation and maintenance of GC. They also involve the processes of B cell antibody affinity maturation, high-frequency mutation, category conversion, and memory B cell formation[5]. Phenotypic markers of Tfh cells include C-X-C motif chemokine receptor 5 (CXCR5), programmed death 1 (PD-1), and inducible co-stimulatory molecule (ICOS). Interleukin (IL)-21, IL-4, and CD40L are crucial effector molecules produced by Tfh cells to induce GC B cell proliferation and differentiation[6]. Abnormalities of Tfh or its effector molecules lead to disorders of the immune system and trigger immune deficiency or autoimmune diseases. It was found that the abnormality of Tfh was associated with the occurrence and progression of SLE by controlling the expressions of CD40L, ICOS, IL-21 and SAP[7].

Casitas B-lineage Lymphoma b (Cblb) is a new gene cloned by Keane et al[8] in 1995. It is the first E3 ubiquitin ligase directly related to T cell activation and tolerance. The E3 ubiquitin ligase Cblb induces ubiquitination of TCR and CD28 signaling proteins and their downstream signaling protein molecules. Hence, it negatively regulates the activation of T cell and induces T cell immune incompetence[9].

T cells have the effect on maintaining the balance between immune tolerance, immune activation and autoimmunity. It was demonstrated that Cblb knockout ($Cblb^{-/-}$) mice either developed spontaneous autoimmunity or had exacerbated immune-mediated pathology in autoimmunity models[10]. Cblb, which acts as a limiting point for T cell activation, is a potential molecular target for the treatment of autoimmune diseases. Gomez-Martin et al[11] have demonstrated that the expression of Cblb is downregulated in T cells from patients with SLE. Hutloff et al[12] showed that the distribution and number of Tfh were abnormal in peripheral blood of patients with SLE. In previous studies, we have established B6-lpr. $Cblb^{C373A}$ mice model by C57BL/6 lpr (B6-lpr) mice crossing with $Cblb^{C373A}$ [Cblb gene (C373A) mutgeneration, inactivating Cblb E3 ubiquitin ligase] mice. The mice showed symptoms of lupus-like earlier. The autoantibody titer and LN performance were more serious than those of B6-lpr mice, which was accompanied with malignant clones of CD4+CXCR5+Bcl-6+ Tfh cells. However, whether Cblb is involved in the development of LN is still unclear. Therefore, in this study, the correlation between Cblb and Tfh, and the potential role of Cblb in the progression of LN were further evaluated.

Materials and Methods

Antibodies and Reagents

The following reagents were obtained from BD Biosciences (San Jose, CA, USA), including anti-ICOS, anti-CD4, anti-PD-1, anti-CXCR5, anti-Bcl-6, recombinant mouse IL-2, purified anti-CD3 (Clone 145-2C11), and anti-mouse CD28 (37.51). Antibodies against CBLB (G-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and pancreatic enzymes were obtained from Invitrogen (Carlsbad, CA, USA). Rabbit anti-human IgG and rabbit anti-mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish-peroxide (HRP) conjugated secondary antibodies for Western blot were purchased from the Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and the secondary antibodies for immunofluorescence were purchased from GBI (Mukilteo, WA, USA). Enzyme linked immunosorbent assay (ELISA) kits for mouse IgG (88-50400) were purchased from eBioscience (San Diego, CA, USA). ELISA kits for anti-ANA and anti-dsDNA [total (A+G+M) (5110)] were purchased from Alpha Diagnostic International Inc. (San Antonio, TX, USA). The enhanced chemiluminescence kit for Western blotting was obtained from GE Healthcare (Buckinghamshire, UK). Mouse neutrophil isolation kit, monocyte isolation kit, and CD45 microbeads (mouse) were purchased from Miltenyi Biotecnology (San Diego, CA, USA). Histopaque 1119 (Sigma-Aldrich, St. Louis, MO, USA; 11191), Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA; 10771), and anti-Flag (M2) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Clinical Samples

Ten patients diagnosed with SLE fulfilling American College of Rheumatology (ACR'97) and/or Systemic Lupus International Collaborating Clinics (SLICC) classification criteria in our hospital from July 2016 to October 2017 were included[13]. All patients with an average age of 31.2 years old were diagnosed with type IV lupus nephritis by renal biopsy. The patients with

heart, brain, kidney or metabolic diseases were excluded. 24 h urine protein, serum creatinine, and BUN in patients were measured. The Tfh ($CD4^+CXCR5^+PD\text{-}1^+$) cells in peripheral blood were analyzed by flow cytometry. The written informed consent was obtained from all the subjects before the study. The use of human subjects was approved by the Medical Ethics Committee (MEC) of Central South University. Research content and methods complied with the specifications and requirements of the MEC.

Animal Model

C57BL/6 (B6) mice and B6-lpr mice were purchased from Slac Animal Laboratory (Shanghai, China). $Cblb^{-/-}$ mice were provided by Prof. Lu (from the Guangzhou Institute of Respiratory Health). lpr.$Cblb^{-/-}$ mice were created by crossing B6-lpr mice to $Cblb^{-/-}$ mice. All animals were maintained on a C57BL/6 background. In the whole research process, they were given free access to commercial standard mouse fodder (Hunan Shi Lai Ke Jing Da Experimental Animal Co. Ltd., Hunan, China) and tap water. They were housed in stability condition at room temperature with a 12 h light/dark cycle. Mice were divided into 4 groups (10/group): wild type (WT) , lpr, $Cblb^{-/-}$ and lpr.$Cblb^{-/-}$. Urine protein was monitored once every four weeks from the fourth week of age. Two mice from each group were sacrificed at the age of 4, 8, 12, 16, and 20 weeks, respectively. Left ventricular blood was collected and tested for serum creatinine, BUN, dsDNA, ANA and IgG. The Tfh ($CD4^+CXCR5^+PD\text{-}1^+$) cells from peripheral blood of mice were analyzed by flow cytometry. Half of the kidneys were fixed in 10% neutral-buffered formalin for Hematoxylin eosin (H&E) and periodic acid-Schiff (PAS) staining. The remaining half was frozen in liquid nitrogen for immunofluorescence.

Histopathological Examination

Kidney tissues of mice were fixed in 10% neutral-buffered formalin, dehydrated in graded alcohol, and embedded in paraffin. Paraffin sections (3-5 μm thick) were stained with H&E and PAS to observe the glomeruli pathological changes.

Immunofluorescence

Kidney tissues of mice were frozen in cryobiolgical inventory system. The freezing tissues were incised to determine IgG deposits in the kidney. The slice was sealed by PBS containing 10% normal goat serum at room temperature for 1 h. It was incubated overnight with IgG monoclonal antibody, followed by incubation with fluorescin isothiocyanate (FITC)-conjugated goat anti-mouse IgG at 37°C for 1 h. The tissues were imaged and measured by the fluorescence microscope.

Flow Cytometry

Tfh cells were collected from kidney from mice or peripheral blood from patients and mice. After centrifugation of tissue mixture or whole blood, supernatant was discarded and RBC lysis buffer was added to lyse erythrocyte. After another centrifugation and supernatant discarding, the cells were washed with PBS for 3 times. The cells at 1×10^6/tube were stained with anti-CXCR5, anti-CD4, and anti-Bcl-6 for 30 min. CXCR5+Bcl-6+ cells were detected using flow cytometry with a CD4+ gate. The cells were washed and acquired immediately on BD LSR Fortessa (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry data were analyzed by FlowJo (version 10.2; Tree Star, Ashland, OR, USA).

Cell Culture and Activation of Tfh Cells

Peripheral T cells from naive WT and lpr mice or from healthy volunteers and patients with LN were isolated (purity Stare). The cells were washed and acquired immediately on BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA). The purified $CD4^+$ T cells were plated at 2×10^6 cells/ml in 24-well flat-bottom plates coated with purified combined anti-CD3 (0.5 μg/ml). Simultaneous additional IL-21 (5 ng/ml) were used to stimulate the differentiation of CD4+ T cells into Tfh cells. Transfection was performed at 30-50% cells confluence.

Transient Transfection and Gene Silencing

Cells from lpr mice or patients with LN were transiently transfected with pEX2-Cblb or pEX2 using Lipofectamine 2000, according to the manufacturer's instructions. Cells from WT mice or healthy volunteers were transfected with Cblb-specific siRNA or control siRNA by Lipofectamine 2000. For Cblb knockdown, silencer select predesigned siRNA for Cblb (sense 5'-UGCCAAGCUUCAGUUGAAATT-3', antisense 5'-UUUCAACUGAAGCUUCGG ATT-3') was used. Silencer negative control siRNA without mammalian homology was used as negative control (control siRNA). Transfected cells were

co-cultured with B cells under the condition of *Staphylococcus* enterotoxin. After 24-48 h, cells were harvested and total lysates were subjected to Western blot analysis. The culture solution was harvested to test IgG by ELISA.

Protein Extraction and Western Blot Analysis

Total proteins from Tfh cells were transfected and collected. Cell lysate containing 20 μg total proteins was separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under the reducing condition, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated overnight at 4°C with primary antibodies. Then, they were incubated 1 h at room temperature with HRP conjugated secondary antibodies. Protein were developed by enhanced chemiluminescence. The bands were scanned on a flatbed scanner and images were imported into Image Studio Lite (version 5.2.5; LI-COR Biosciences, St. Louis, MO, USA). Individual bands were manually identified and band intensity was determined by Image Studio. Relative intensities were calculated and plotted in GraphPad Prism (version 6.0, GraphPad Software, San Diego, CA, USA). The results were expressed as the percentage change in the mean band density compared with the control values.

ELISA

The supernatants harvested at 48 h after transfection were homogenized, and the supernatants were recovered following centrifugation at 15,000 g for 20 min at 4°C after transfection.

Statistical Analysis

Statistical analysis was performed using SPSS 23.0 Software (SPSS 23.0, IBM Corp., Armonk, NY, USA). The results were expressed as means ± standard deviation (SD). One-way ANOVA was applied to compare the differences between three or more groups. $p<0.05$ was considered to be statistical significance.

Results

Peripheral Tfh Cells Were Augmented and the Expression of Cblb in T Cells was Downregulated in LN Patients

The 24 h urine protein and serum creatinine were significantly higher in patients with LN than those in healthy volunteers ($p<0.05$) (Table I). The peripheral Tfh cells were higher in patients with LN than those in health volunteers ($p<0.05$) (Figure 1A). Notably, the expression of Cblb was reduced in Tfh cells in patients with LN, while the Tfh cells was significantly increased ($p<0.05$) (Figure 1B).

Deletion of Cblb Aggravated Kidney Damage in lpr Mice

Lpr.Cblb$^{-/-}$ mice and lpr mice showed an increase of urine protein levels compared with WT mice ($p<0.05$), while the urine protein levels were significantly higher in lpr.Cblb$^{-/-}$ mice than those in lpr mice ($p<0.05$). There was no significant difference in BUN levels among the four groups until mice were 20 weeks old. Serum creatinine levels of lpr.Cblb$^{-/-}$ mice and lpr mice showed an increase trend since 12 weeks age of mice. At 20 weeks old, BUN and serum creatinine levels in lpr.Cblb$^{-/-}$ mice were higher than mice in other groups ($p<0.05$) (Figure 2A). It was found that ANA and anti-dsDNA levels in lpr.Cblb$^{-/-}$ mice and lpr mice were increased compared to WT mice at different times ($p<0.05$). Meanwhile, the level of lpr.Cblb$^{-/-}$ mice were higher than lpr mice ($p<0.05$) (Figure 2B). Renal histopathological examination in mice showed thickening of glomerular capillary basement membrane, proliferation of

Table I. Basic characteristic of the LN patients and healthy volunteers.

	Healthy volunteers	LN patients
Age (years)	31.00 ± 7.64	31.20 ± 11.70
Lupus Activity Measure score	—	14.50 ± 2.46
Urine protein (g/24 h)	0.08 ± 0.03	5.11 ± 1.73*
BNU (mol/L)	4.97 ± 1.01	5.77 ± 2.24
Cr (μmol/L)	56.1 ± 12.74	82.6 ± 18.53*

*_vs_. Healthy volunteers, $p < 0.05$.

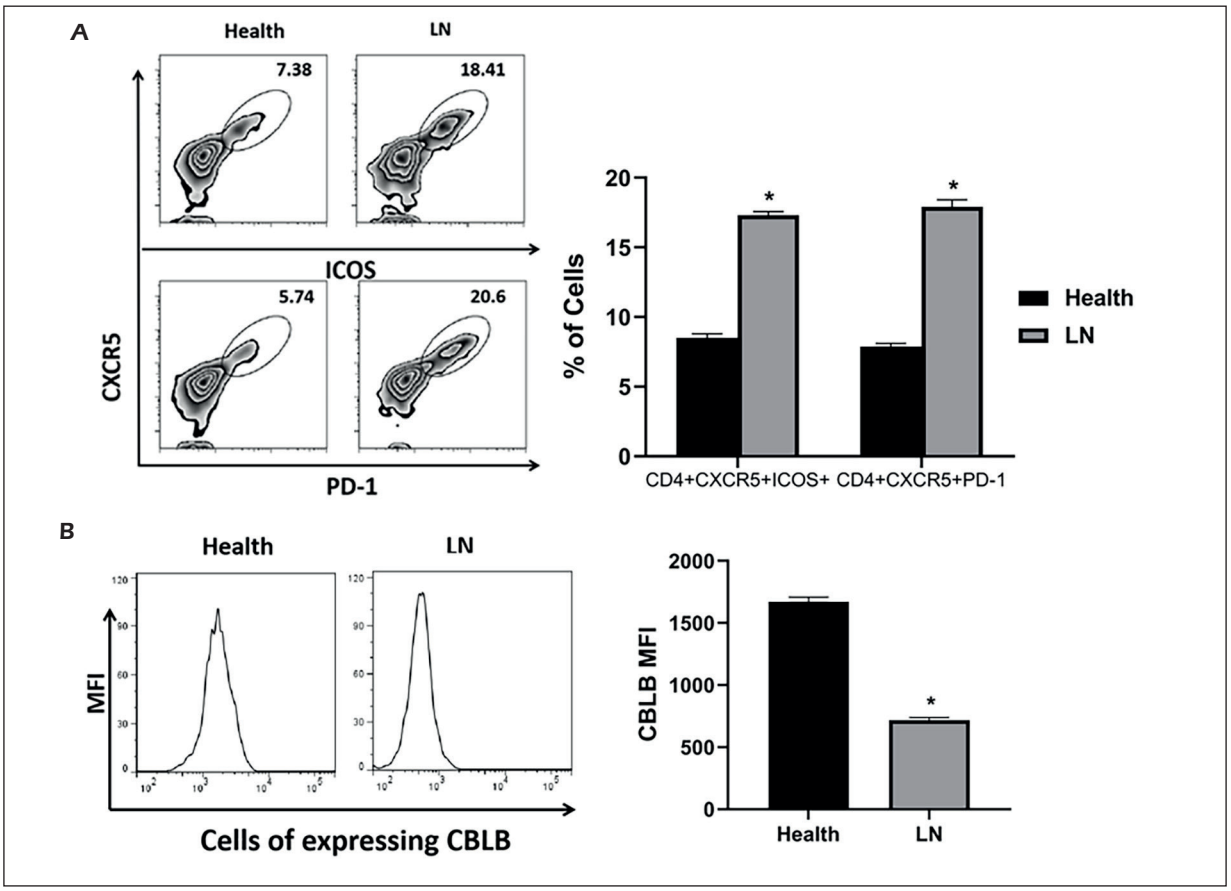

Figure 1. The expression of CBLB in Tfh cells was down-regulated in LN patient, while the peripheral Tfh cells were increased. **A,** The patients were divided two groups (10/group), LN patients and healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were harvested from peripheral blood of the patients and healthy volunteers by density-gradient centrifugation on Ficoll-Paque Plus. CD4+CXCR5+ICOS+PD-1+ T cells were measured by flow cytometry. **B,** Tfh cells were harvested and relative CBLB intensity levels were quantified by flow cytometry. Data expressed as mean ± SD, *p<0.05 *vs.* healthy volunteers, analyzed by unpaired two-tailed Students t-test.

mesangial cells and endothelial cells in lpr.Cblb $^{-/-}$ and lpr mice. However, renal pathological alteration in lpr.Cblb$^{-/-}$ mice were more severe than lpr mice (Figure 2C).

Deletion of Cblb Increased Tfh Cells in Peripheral Blood in lpr Mice

The number of peripheral Tfh cells was significantly higher in lpr.Cblb$^{-/-}$ and lpr mice than that in WT mice, and there were more peripheral Tfh cells in lpr.Cblb$^{-/-}$ mice than lpr mice (p<0.05, respectively) (Figure 3A). Meanwhile, the IgG levels in peripheral blood from lpr. Cblb$^{-/-}$ and lpr mice were also increased, and the IgG levels were higher in lpr.Cblb$^{-/-}$ mice compared with lpr mice (p<0.05) (Figure 3B). We further detected the deposition of IgG in renal tissue sections by immunofluorescence (Figure 3C). The expression of IgG was increased in Cblb$^{-/-}$ mice and lpr mice when compared to WT mice. Moreover, the expression of IgG in lpr. Cblb$^{-/-}$ mice was higher than lpr mice (p<0.05) (Figure 3D).

Cblb Led to the Activation of B Cells Via Negatively Regulation on Tfh

The expressions of Cblb were reduced and IgG levels were increased in siRNA group when compared to control group (p<0.05). Meanwhile, the expressions of Cblb were increased and IgG levels were decreased in plasmid group than control group (p<0.05) (Figure 4).

Discussion

SLE sometimes tends to be refractory to traditional treatments. It also tends to be life threat-

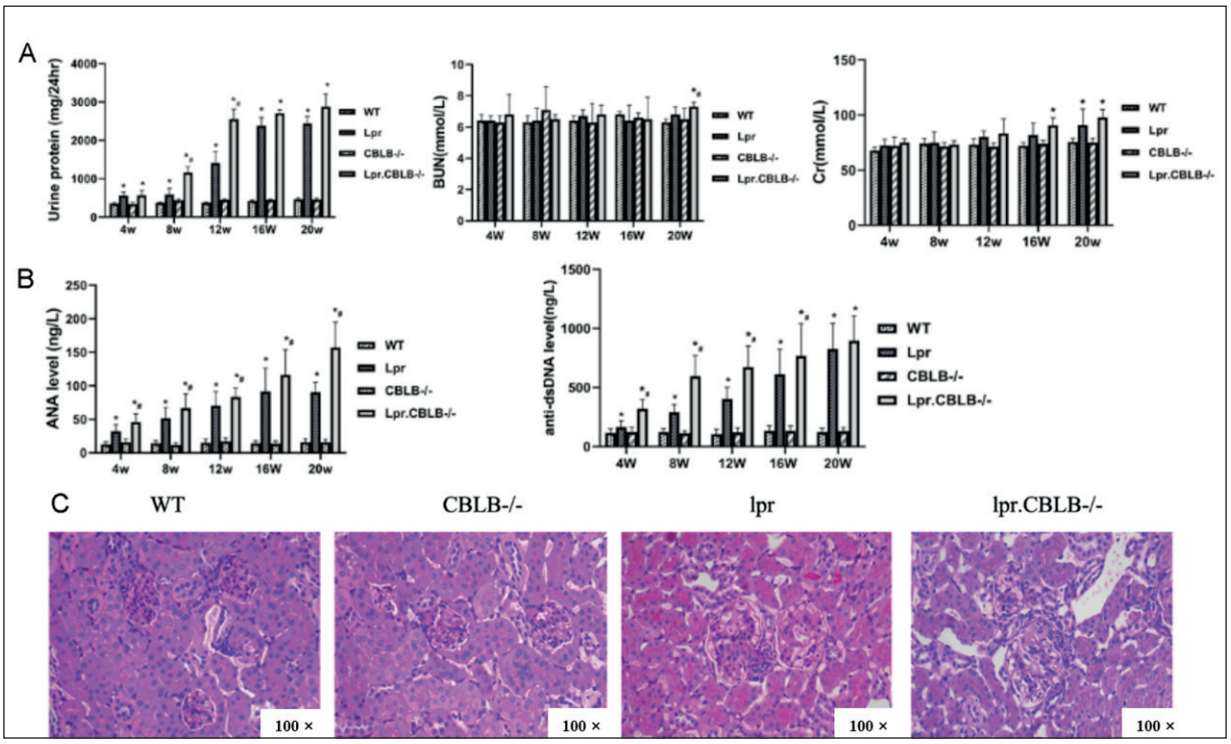

Figure 2. Deletion of CBLB aggravates kidney damage in lpr mice. **A,** 24h-urine protein, BUN, serum creatinine levels were monitored at 4, 8, 12, 16, 20 weeks old mice. 24ch urine protein was measured by Coomassie blue staining. BUN and serum creatinine levels were measured by ELISA. **B,** ds-DNA, ANA levels were detected by ELISA when mice were 4, 8, 12, 16, 20 weeks old. **C,** 8 mice from each group were sacrificed at 8 weeks old. Kidney tissues sections were stained with PAS to observe the variation of pathology with a microscope under 10 × magnification. Data expressed as mean ± SD, *$p<0.05$ *vs*. WT; $^{\#}p<0.05$ *vs*. lpr, analyzed by One-way ANOVA.

ening, especially when major vital organs are invaded. The results presented in this study have identified that peripheral Tfh cells are increased in patients with LN. We speculated that Tfh cells played important roles in LN. Consistent with our results, Feng et al[14] have also provided evidences that there is a relationship between abnormality of Tfh and the development of SLE. Kitaura et al[15] have shown that the amount of circulating Tfh cells is increased in 14 patients of 46 patients with SLE, while it is not increased in the control group.

It was found that the distribution and number of Tfh cells were abnormal in peripheral blood of patients with SLE. The serum IL-21 levels were anomalously increased. Germinal center (GC)-like structures and inducible costimulators (ICOS+) T cells could be found in the kidney tissues of patients, and local IgG deposition and hyper-IgG lead to excessive differentiation and proliferation of local plasma cells and memory cells[7,16,17]. Jang et al[18] showed that human bone marrow-derived mesenchymal stem cells directly suppressed the *in vitro* differentiation of naive CD4+ T cells toward Tfh cells in a contact-dependent manner. These results suggested that MSCs attenuated lupus nephritis by suppressing the development of Tfh cells. MSCs also attenuated the subsequent activation of humoral immune components, which was also in accordance with our results.

In the current report, we found that the expressions of Cblb were down-regulated in Tfh cells of patients with LN. Cblb has been shown to be a key negative regulator of T cell activation. Our previous studies have shown that the gene expression of Cblb is strictly regulated by CD28 and CTLA-4, which plays an important role in the activation and tolerance of T cells. Therefore, we speculated that the increase of Tfh cells was regulated by decreased expression of Cblb in patients with LN[19,20]. Cblb was considered to be related with SLE. Cblb mutation could exacerbate immune responses of antibody and increase formation of GC in mice. Other researches[11,15] also showed that the

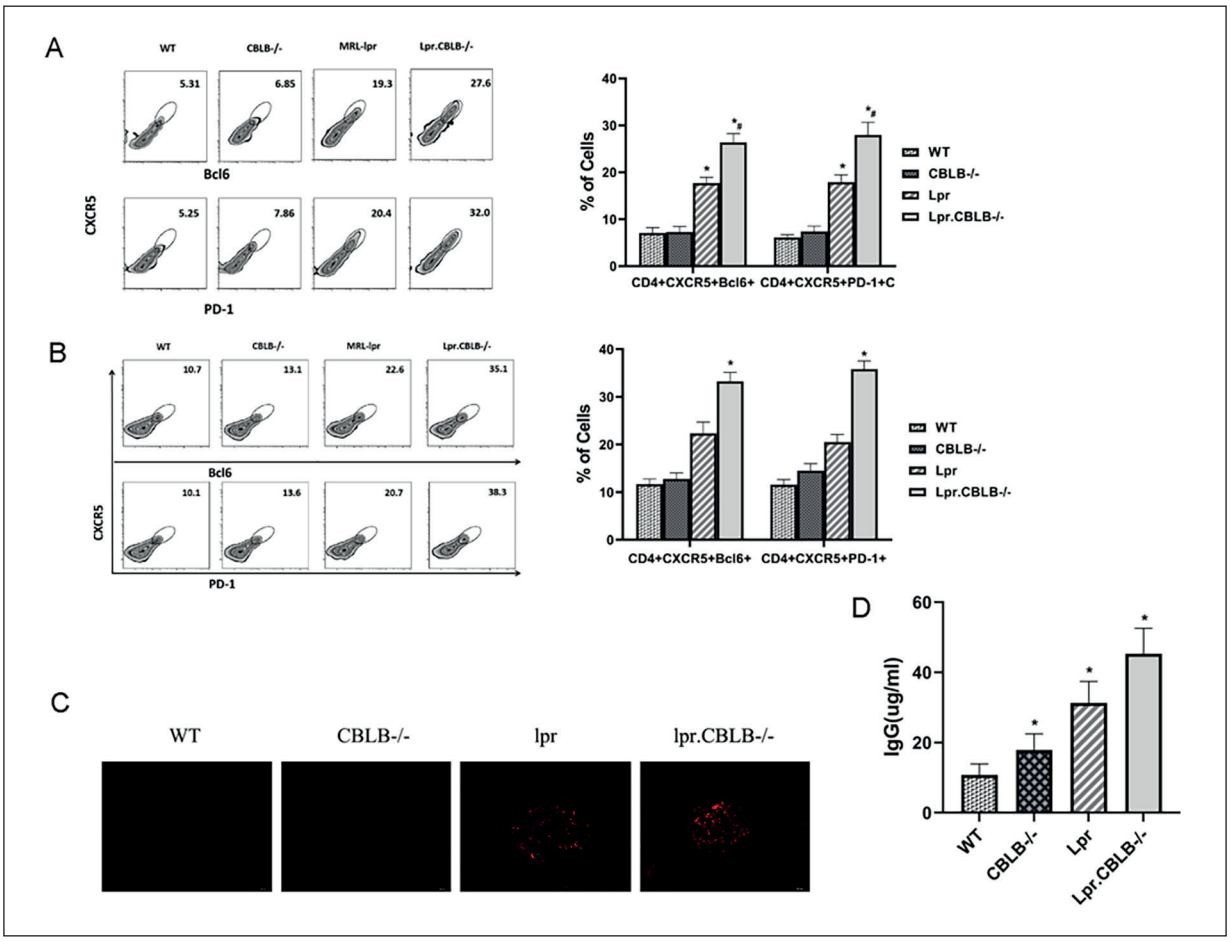

Figure 3. Tfh or like-Tfh cells and IgG expression in peripheral blood and renal in mice. **A,** Mice were divided into 4 groups (10/group): WT, CBLB$^{-/-}$, lpr, lpr.CBLB$^{-/-}$. 8 mice from each group were sacrificed at 8 weeks old. CD4+CXCR5+Bcl6+PD-1+ T cells from peripheral blood of mice were counted with flow cytometry. **B,** 8 mice from each group were sacrificed at 8 weeks old. CD4+CXCR5+Bcl6+PD-1+ T cells like-Tfh from renal of mice were counted with flow cytometry. **C,** 8 mice from each group were sacrificed at 8 weeks old. The kidney tissues were frozen to test the deposition of IgG by immunofluorescence (100 × magnification). **D,** Left ventricular blood was collected and IgG was measured by ELISA. Data expressed as mean ± SD, *p<0.01 *vs.* WT; $^{\#}p$<0.01 *vs.* lpr, analyzed by One-way ANOVA.

deficiency of Cblb would either induce spontaneous autoimmunity disease or exacerbate immune-mediated pathology in autoimmunity models. In order to identify the role of Cblb in patients with LN, we have used the lpr.Cblb$^{-/-}$ mice built by lpr mice crossing with Cblb$^{-/-}$ mice. Our data showed that lpr.Cblb$^{-/-}$ mice had more serious nephropathological changes than B6-lpr mice.

The deletion of Cblb will aggravate pathological changes in LN. Meanwhile, we also found peripheral Tfh cells were increased in lpr.Cblb$^{-/-}$ mice and lpr mice, while the number of peripheral Tfh cells in lpr.Cblb$^{-/-}$ mice was higher than lpr mice. Furthermore, serum IgG and the deposition of IgG in kidney tissue were significantly increased in lpr.Cblb-/- mice compared to lpr mice. It was found that the increase of Tfh would lead to the increase of pathological GC, which would further lead to organ damage, such as lupus nephritis, etc[21]. In this investigation, we found that deletion of Cblb would aggravate kidney damage in lpr mice due to the decrease of suppression to Tfh.

We have further investigated whether alteration of Cblb expression in Tfh cells in patients with LN was consisted with the experiment results in lpr mice. Cblb expression was modulated by transfecting siRNA and overexpression plasmid approach in Tfh cells of patients with LN *in vitro*. Tfh and IgG were reduced when Cblb expression was increased. Moreover, the Tfh and IgG were

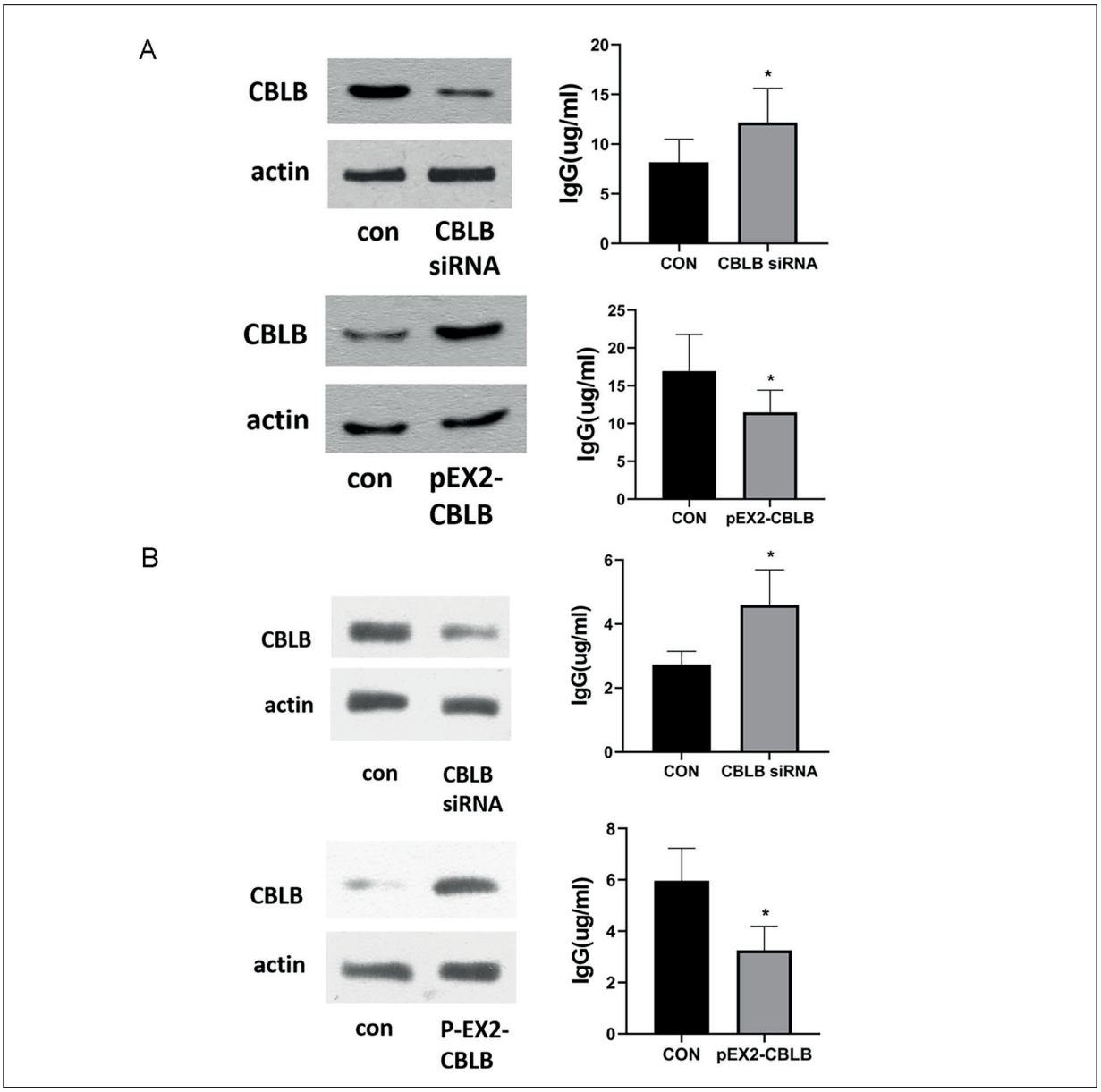

Figure 4. Cblb induced the activation of B cells via regulated Tfh. **A,** Peripheral T cells were isolated from WT and lpr mice on T cell enrichment columns, and stimulated with anti-CD3 and anti-CD28. Cells from lpr mice were transiently transfected with pEX2-Cblb using Lipofectamine 2000. Cells from WT mice were transfected with Cblb specific siRNA by using Lonza nucleofector reagent. Transfected cells were co-cultured with B cells under the condition of *Staphylococcus* enterotoxin. Cells were then harvested and total lysates were subjected to Western blot analysis. The culture solution was harvested to detect IgG by ELISA. **B,** The peripheral T cells from patients with LN and healthy volunteers were isolated on T cell enrichment columns, and stimulated with anti-CD3 and anti-CD28. Cells from patients with LN were transiently transfected with pEX2-Cblb using Lipofectamine 2000. Cells from healthy volunteers were transfected with Cblb specific siRNA using Lonza nucleofector reagent. Transfected cells were co-cultured with B cells under the condition of *Staphylococcus* enterotoxin. Cells were then harvested and total lysates were subjected to western blot analysis. The culture solution was harvested to detect IgG by ELISA. Data expressed as mean ± SD, **p*<0.05 *vs*. con, analyzed by unpaired two-tailed Student's *t*-test.

increased when Cblb expression was reduced. We modulated the expression of Cblb in Tfh cells of lpr mice *in vitro* simultaneously, which was used as a contrast experiment for the patients. The results in mice were consistent with the results in the patients. Therefore, we demonstrated that the Cblb played a key role in the occurrence and development of lupus nephritis. As a negative regulator

of Tfh, the downregulation of Cblb would lead to malignant clones of Tfh cells in patients with LN. Deregulation of Tfh activities could contribute to a pathogenic autoantibody production which played an important role in the progress of LN.

Conclusions

The findings of the present study showed that Cblb was a major regulator for Tfh which activated B cells and mediated the occurrence of lupus nephritis. Overexpression of Cblb might be one of the mechanisms, by which a blockade of the humoral immunity process conferred renal protective effects.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgment

This study was funded by the National Natural Science Foundation of China (Grants No. 81600538).

Ethical Approval

The written informed consent was obtained from all the subjects before the study. The use of human subjects was approved by the Medical Ethics Committee (MEC) of the first affiliated hospital of Guangzhou Medical University.

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