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Calcineurin B1 subunit in human peripheral blood mononuclear cells and its role in idiopathic membranous nephropathy

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Abstract

The immune responses involved in the pathogenesis of idiopathic membranous nephropathy (IMN) have not been fully understood. Calcineurin, a key signaling enzyme in T-cell activation, may be implicated in IMN. The present study aimed to investigate the role of calcineurin B1 subunit (CnB1) in IMN and the potential mechanism.

A total of 59 biopsy-proven IMN patients and 28 healthy controls were recruited. The CnB1 expression in human peripheral blood mononuclear cells (PBMCs) was assessed by Western blotting. Knockdown and overexpression of CnB1 in Jurkat T cell line were achieved by small interference RNA (siRNA) transfection and lentiviral transduction, respectively.

It was found that PBMCs CnB1 expression was significantly increased in IMN patients ($P = .002$), but unrelated to the severity and prognosis of IMN. Knockdown of CnB1 in Jurkat cells inhibited the nuclear factor of activated T cells (NFAT)-regulated gene expression required for T-cell activation.

Our study suggested the potential role of CnB1 in the occurrence of IMN. The mechanism maybe involved the effect of CnB1 on the T-cell activation mediated by calcineurin-NFAT signaling.

Abbreviations: CnB1 = calcineurin B1 subunit, CNIs = calcineurin inhibitors, GFP = green fluorescent protein, GM-CSF = granulocyte-monocyte colony-stimulating factor, IL-2 = interleukin-2, IMN = idiopathic membranous nephropathy, LV = lentiviral vector, NFAT = nuclear factor of activated T cells, PBMCs = peripheral blood mononuclear cells, PCR = polymerase chain reaction, PLA2R = phospholipase A2 receptor, PMA = phorbol 12-myristate 13-acetate, siRNA = small interference RNA, THSD7A = thrombospondin type-1 domain-containing 7A.

Keywords: calcineurin B1 subunit, calcineurin inhibitors, idiopathic membranous nephropathy, NFAT, T-cell activation

1. Introduction

Idiopathic membranous nephropathy (IMN) is a leading cause of adult nephrotic syndrome. Our understanding of its pathogenesis has greatly increased in recent years. It is primarily an autoimmune glomerular disease mediated by autoantibodies against M-type phospholipase A2 receptor (PLA2R), thrombospondin type-1 domain-containing 7A (THSD7A), or other unidentified autoantigens.[1,2] The discovery of these autoantibodies suggests a pathogenic role of B cells in IMN. Follicular helper T cells, a subset of CD4^{+} T cells, are known to regulate the programming of B-cell immunity including the terminal differentiation of B cells into antibody-producing cells.[3] Previous studies have found that activated follicular helper T cells in peripheral blood contribute to the pathogenicity of IMN.[4,5] However, the precise mechanisms of T-cell activation in IMN remain unclear.

Calcineurin-nuclear factor of activated T cells (NFAT) pathway is thought to play a critical role in T-cell activation.[6] In T cells, increased intracellular Ca^{2+} levels activate the phosphatase activity of calcineurin, leading to the dephosphorylation of NFAT in the cytoplasm.[7] Dephosphorylated NFAT translocates to the nucleus and induces the gene transcription of cytokines including interleukin-2 (IL-2) and granulocyte-monocyte colony-stimulating factor (GM-CSF), which activates T cells and promotes immune responses.[8]

Calcineurin is a phosphatase consisting of a catalytic subunit and a regulatory subunit. In mammals, there are 2 isoforms of calcineurin regulatory subunit (CnB1 and calcineurin B2 subunit). Calcineurin B2 subunit is expressed only in testes, whereas CnB1 is ubiquitously expressed and indispensable for maintaining calcineurin activity.[9,10]

This study focused on the CnB1 in human peripheral blood mononuclear cells (PBMCs) to explore its role in the pathophysiologic process of IMN and the potential mechanism. It also examined its clinical value of predicting the severity and prognosis of IMN.

2. Materials and methods

2.1. Study population

A total of 59 biopsy-proven IMN patients at the Department of Nephrology, Huashan Hospital, Fudan University from April 2015 to December 2017 were included in the study. Secondary causes such as lupus and hepatitis were excluded to confirm IMN. The control group consisted of 28 healthy individuals with matched age and sex. The study was approved by the ethics committee of Huashan Hospital, Fudan University. Written informed consent was obtained from all participants.

2.2. Sample preparation

Fasting venous blood of each participant was collected into the EDTA-anticoagulant tube. PBMCs were isolated from each blood sample by density gradient centrifugation using the lymphocyte separation medium (Corning, USA) according to the manufacturer's protocols.

2.3. Western blotting

Proteins were extracted from cells using RIPA lysis buffer (Beyotime, China). Then proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, USA). The membranes were incubated with the primary antibody against human CnB1 (MAB1348; R&D Systems, USA) overnight at 4°C, followed by the reaction with the horseradish peroxidase-conjugated secondary antibody for an hour at room temperature. The signal was detected using the chemiluminescence reagent (Millipore). CnB1 expression levels were quantified by densitometry using ImageJ software.

2.4. Cell culture

Jurkat cells (an immortalized cell line of human T lymphocytes) were cultured in the RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin (HyClone), and 100 μg/mL streptomycin (HyClone) at 37°C in a humidified atmosphere with 5% CO_2.

2.5. RNA interference

Small interference RNA (siRNA) of human CnB1 gene and scrambled siRNA were designed and synthesized by Biotend (Shanghai, China). The siRNA sequences were as follows: CnB1 siRNA (sense: 5′-GAUACACAGUUACAGCAAA-3′, antisense: 5′-UUUGCUGUAACUGUGUAUC-3′) and scrambled siRNA (sense: 5′-UUCUCCGAACGUGUCACGU-3′, antisense: 5′-ACGUGACACGUUCGGAGAA-3′). Transfection was performed using the Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) following the manufacturer's instructions.

2.6. Lentiviral vector transduction

Lentiviral vector (LV) expressing the DNA fragment encoding green fluorescent protein (GFP)-tagged full-length human CnB1 (LV-CnB1) was constructed, packed, and purified. As a control, LV expressing GFP alone (LV-control) was also generated. Transduction was performed according to the manufacturer's protocols.

CnB1 expression in the PBMCs of controls and IMN patients.

	Controls	IMN patients	*P*
Number	28	59	
Age, y	58.5 (34, 69)	51 (39, 58)	.12
Sex, male/female	16/12	44/15	.10
CnB1 expression	0.27 (0.12, 0.45)	0.52 (0.29, 0.68)	.002[*]

CnB1 = calcineurin B1 subunit, IMN = idiopathic membranous nephropathy, PBMCs = peripheral blood mononuclear cells.

[*] $P < .05$

2.7. Quantitative real-time polymerase chain reaction

After transfection with siRNAs or transduction with LVs, Jurkat cells were cultured for 3 hours in the presence or absence of 20 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, USA) and 1 μg/mL ionomycin (Sigma-Aldrich). Then the cells were collected to extract total RNA using the TRIzol Reagent (Invitrogen). Reverse transcription was completed using the PrimeScript RT Master Mix Kit (RR036A, TaKaRa, Japan). Quantitative real-time polymerase chain reaction (PCR) was performed using the SYBR Premix Ex Taq Kit (RR820A, TaKaRa). The primers were as follows: CnB1 (forward: 5′-GT-GCTCACACTTTGATGCGG-3′, reverse: 5′-TCTCCATTCC-CATCTGTGTCG-3′), IL-2 (forward: 5′-GACCCAGGGACT-TAATCAGCA-3′, reverse: 5′-AATGGTTGCTGTCTCATCA-GC-3′), GM-CSF (forward: 5′-GCATGTGAATGCCATCCA-GG-3′, reverse: 5′-CCTGCTTGTACAGCTCCAGG-3′), and GAPDH (forward: 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse: 5′-GAAGATGGTGATGGGATTTC-3′). The relative mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as the internal reference gene.

2.8. Detection of IL-2 and GM-CSF production

After transfection with siRNAs or transduction with LVs, Jurkat cells were stimulated with PMA (20 ng/mL)/ionomycin (1 μg/mL). The supernatant samples were collected 24 hours after the stimulation and stored at −80°C until use. Production of IL-2 and GM-CSF in the supernatant samples was measured using the ELISA kits (Anogen, Canada).
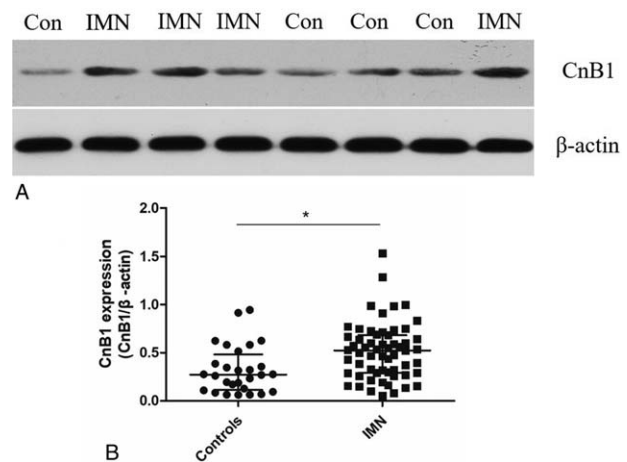
Figure 1. Elevated PBMCs CnB1 expression in IMN patients. (A) Representative Western blotting results. Con = controls. (B) Quantification of CnB1 expression normalized to β-actin. Controls: n = 28, IMN: n = 59. [*]P = .002.

2.9. Statistical analysis

Data were expressed as mean ± SD or median (interquartile range). Unpaired t test or Mann–Whitney test was performed for continuous variables and χ^2 test was used for categorical variables. Statistical significance was defined as $P < .05$. All data analyses were performed using Stata 10.0 software.

3. Results

### 3.1. Elevated PBMCs CnB1 expression in IMN patients

To investigate the potential role of CnB1 in IMN, we first tested the PBMCs CnB1 expression in 59 IMN patients and 28 healthy controls. Western blotting showed that it was significantly increased in the patients when compared with the controls ($P = .002$), suggesting that CnB1 in PBMCs may have an impact on developing IMN (Table 1, Fig. 1).

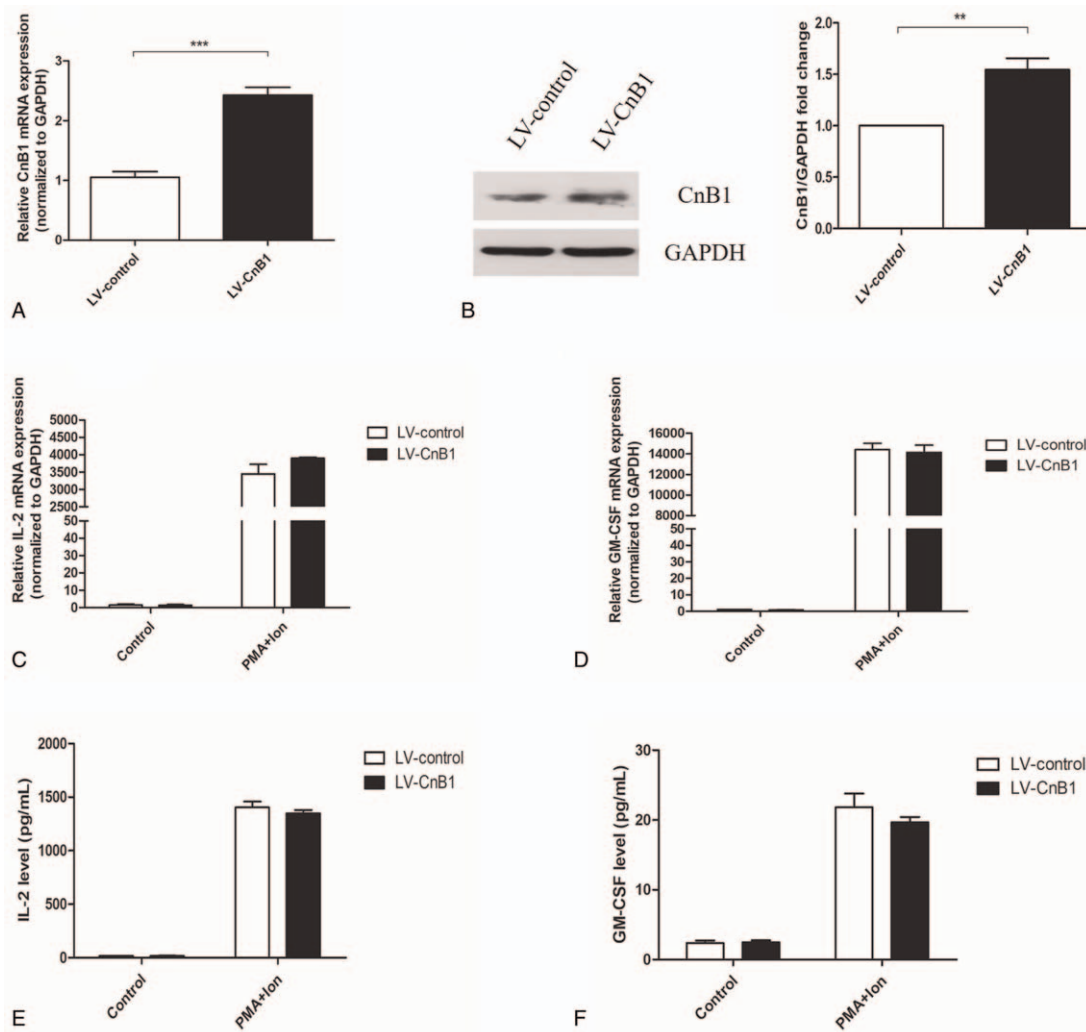### 3.2. Knockdown of CnB1 and its effect on the NFAT-regulated gene expression in Jurkat cells

To explore the potential mechanism, we put forward the hypothesis that CnB1 influences the occurrence of IMN through its effect on the T-cell activation mediated by calcineurin-NFAT signaling.

To test this hypothesis, Jurkat cells were transfected with CnB1 siRNA or scramble siRNA. Real-time PCR showed that the CnB1 mRNA expression level was significantly lower in CnB1-siRNA cells than in scramble-siRNA cells (Fig. 2A). Western blotting demonstrated that the CnB1 protein expression level was downregulated in CnB1-siRNA group (Fig. 2B).

Then PMA/ionomycin were added to induce the activation of calcineurin-NFAT pathway. The expression levels of the NFAT-regulated genes (IL-2 and GM-CSF) were used to reflect the extent of T-cell activation. The mRNA expression levels of IL-2
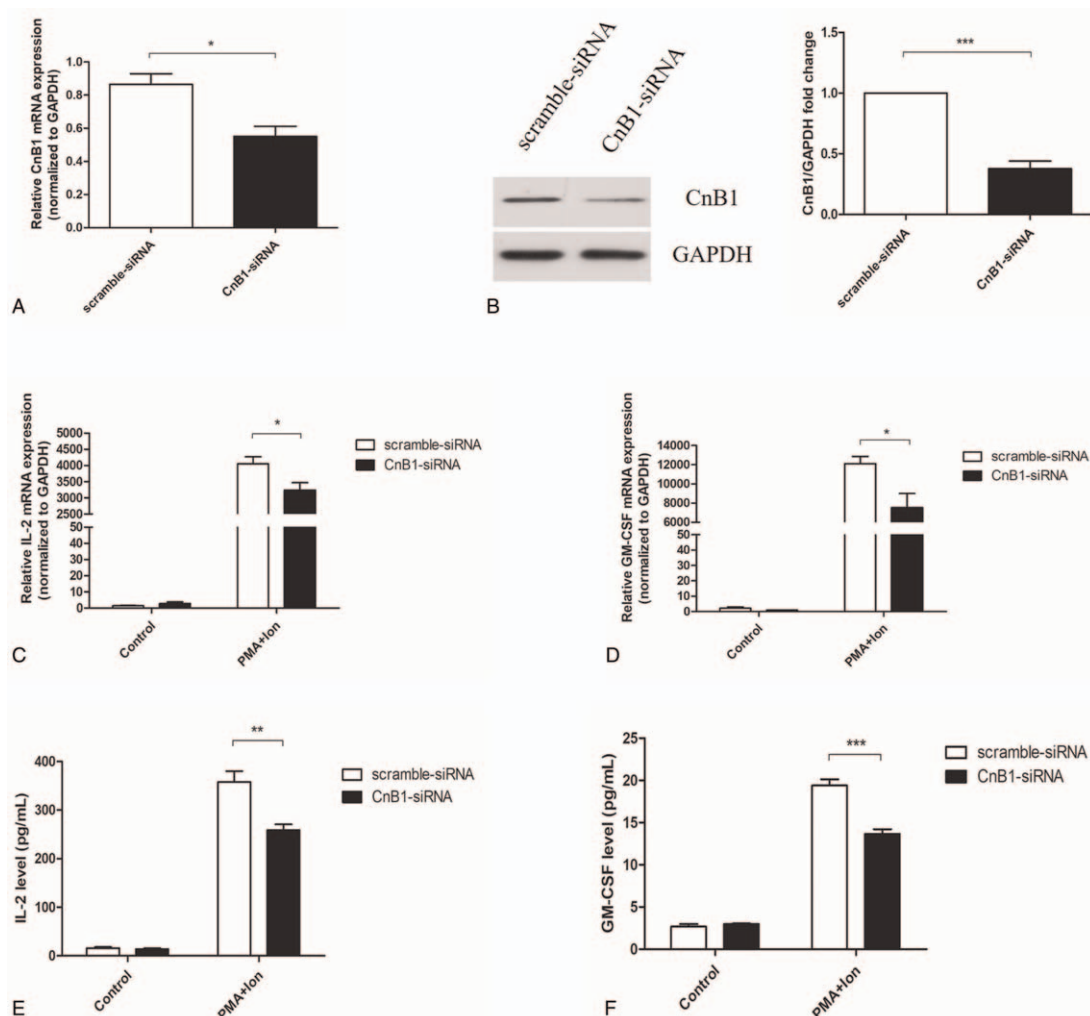
Figure 2. Knockdown of CnB1 and its effect on the NFAT-regulated gene expression in Jurkat cells. Cells were transfected with scramble siRNA or CnB1 siRNA and then stimulated with PMA plus ionomycin. (A) Real-time PCR showed downregulation of CnB1 mRNA in CnB1-siRNA cells (n = 4). (B) Representative Western blotting showed downregulation of CnB1 protein in CnB1-siRNA cells. Quantification of CnB1 fold change was based on 3 repeated experiments. (C, D) Real-time PCR of IL-2 and GM-CSF mRNA was performed 3 h after stimulation (n = 4). (E, F) IL-2 and GM-CSF production in the supernatant of Jurkat cells was assayed 24 h after stimulation (n = 6). Ion = ionomycin. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

Figure 3. Overexpression of CnB1 and its effect on the NFAT-regulated gene expression in Jurkat cells. Cells were transduced with LV-control or LV-CnB1 and then stimulated with PMA plus ionomycin. (A) Real-time PCR showed upregulation of CnB1 mRNA in LV-CnB1 cells (n=4). (B) Representative Western blotting showed upregulation of CnB1 protein in LV-CnB1 cells. Quantification of CnB1 fold change was based on 3 repeated experiments. (C, D) Real-time PCR of IL-2 and GM-CSF mRNA was performed 3 h after stimulation (n=4). (E, F) IL-2 and GM-CSF production in the supernatant of Jurkat cells was assayed 24 h after stimulation (n=6). Ion = ionomycin. $^{**}P < .01$; $^{***}P < .001$.

and GM-CSF in CnB1-siRNA group remarkably decreased compared with scramble-siRNA group (Fig. 2C and D). A significant reduction was observed in the production of IL-2 and GM-CSF when cells were transfected with CnB1 siRNA (Fig. 2E and F). These data indicated that knockdown of CnB1 inhibited the NFAT-regulated gene expression required for T-cell activation.

3.3. Overexpression of CnB1 and its effect on the NFAT-regulated gene expression in Jurkat cells

Subsequently, we tested whether overexpression of CnB1 promoted T-cell activation. Overexpression of CnB1 in Jurkat cells was achieved by lentiviral transduction. As shown in Figure 3A and B, the CnB1 mRNA and protein expression levels were notably increased in the cells transduced with LV-CnB1. After the treatment with PMA/ionomycin, there were no significant differences in the mRNA expression levels of IL-2

and GM-CSF between LV-CnB1 group and LV-control group (Fig. 3C and D). The levels of IL-2 and GM-CSF production were also similar between the 2 groups (Fig. 3E and F). These results revealed that overexpression of CnB1 had no effect on the NFAT-regulated gene expression.

3.4. Relationship between PBMCs CnB1 expression and the clinical parameters in IMN patients before treatment

The IMN patients were divided into 2 groups, low CnB1 expression group and high CnB1 expression group, according to the median of the CnB1 expression levels of the 59 patients. To investigate the clinical significance of CnB1, we compared the clinical measures including 24-h urinary protein, serum albumin and serum creatinine between the 2 groups. As shown in Table 2, the values of the clinical measures were similar in the 2 groups before treatment, indicating that PBMCs CnB1 expression may be irrelevant to the severity of IMN.

Table 2

Baseline characteristics of 59 adult patients with IMN.

	Low CnB1 expression	High CnB1 expression	P
Number	29	30	
Age, y	51 (36, 57)	52.5 (42, 59)	.44
Sex, male/female	23/6	21/9	.41
Proteinuria, g/d	4.42 (1.68, 8.87)	4.66 (3.70, 8.74)	.56
Serum albumin, g/L	25.5 (21.0, 29.0)	24.5 (21.5, 30.6)	.88
Serum creatinine, μmol/L	78.0 (64.0, 85.0)	73.5 (60.0, 85.0)	.56

CnB1 = calcineurin B1 subunit, IMN = idiopathic membranous nephropathy.

3.5. Relationship between PBMCs CnB1 expression and the clinical parameters in IMN patients after supportive therapy for 6 months

Forty-one patients (20 in low CnB1 expression group and 21 in high CnB1 expression group) were treated with supportive care alone for >6 months. Comparisons of the aforementioned clinical measures were made between the 2 groups. The results showed that the values of the clinical measures in low CnB1 expression group were equivalent to those in high CnB1 expression group after supportive therapy for 6 months (Table 3).

3.6. Relationship between PBMCs CnB1 expression and the clinical parameters in IMN patients after immunosuppressive therapy for 6 months

Immunosuppressant drugs including cyclophosphamide and calcineurin inhibitors (CNIs) were used in 29 patients. In low CnB1 expression group, 9 patients were treated with cyclophosphamide, whereas 5 patients were treated with CNIs. In high CnB1 expression group, cyclophosphamide and CNIs were used in 9 and 6 patients, respectively. Likewise, no significant differences were observed in the values of the clinical measures between the 2 groups after immunosuppressive therapy for 6 months (Table 4).

4. Discussion

CNIs are widely used in the treatment of IMN. The monotherapy with tacrolimus which belongs to CNIs has been proven to be a very useful therapeutic approach for IMN patients.[11] However, little is known about the role of calcineurin in the pathogenesis of IMN.

Table 3

Clinical profile of IMN patients with supportive therapy for 6 months.

	Low CnB1 expression	High CnB1 expression	P
Number	20	21	
Age, y	49.5 (34.5, 56.5)	54 (42, 61)	.68
Sex, male/female	17/3	13/8	.10
Proteinuria, g/d	3.61 (2.83, 8.56)	4.90 (3.50, 6.80)	.96
Serum albumin, g/L	32.5 (21.5, 37.0)	27.0 (21.0, 35.0)	.50
Serum creatinine, μmol/L	74.0 (65.0, 87.0)	70.0 (55.0, 88.0)	.66

CnB1 = calcineurin B1 subunit, IMN = idiopathic membranous nephropathy.

Table 4

Clinical profile of IMN patients with immunosuppressive therapy for 6 months.

	Low CnB1 expression	High CnB1 expression	P
Number	14	15	
Age, y	52 (38, 66)	57 (50, 59)	.51
Sex, male/female	10/4	14/1	.12
Proteinuria, g/d	4.92 (1.69, 10.31)	4.41 (1.89, 7.11)	.83
Serum albumin, g/L	28.0 (22.0, 37.0)	31.0 (25.0, 35.0)	.91
Serum creatinine, μmol/L	79.0 (64.0, 85.0)	97.0 (74.0, 129.0)	.07

CnB1 = calcineurin B1 subunit, IMN = idiopathic membranous nephropathy.

IMN is generally considered to be an immune-mediated glomerular disease. The complicated immune responses in IMN have not yet been completely clarified. Some researchers found an elevated CD4+/CD8+ T-cell ratio in PBMCs of IMN patients, showing an imbalance between the CD4+ and CD8+ subsets in IMN.[12,13] Subsequently, Shi et al[4] reported that the frequency of follicular helper T cells and the ratio of ICOS+/PD-1+ follicular helper T cells in peripheral blood were increased in IMN patients. This observation clearly pointed out which subset the elevated CD4+ T cells belong to as well as their activated status. The research of Zhang et al[5] also showed that activated follicular helper T cells in peripheral blood were significantly increased in IMN patients. Moreover, the concentrations of cytokines (IL-2, IL-4, IL-10, and IL-17A) were significantly elevated in the sera of IMN patients. These findings presented here illustrate that activated follicular helper T cells and cytokines take part in the pathogenic process of IMN. However, the mechanisms of T-cell activation and cytokine production in IMN have not been fully elucidated.

Calcineurin is a key component of the signal transduction required for T-cell activation.[14] Studies have shown that calcineurin in T cells is implicated in some autoimmune human diseases and animal models.[15,16] In this study, we first compared the CnB1 expression in PBMCs between IMN patients and healthy controls. It was found that the PBMCs CnB1 expression level in the patients was significantly higher than that in the controls, suggesting its potential role in developing IMN.

NFAT, an inducible nuclear factor, is the main target of calcineurin.[17] Considering its importance in T-cell activation,[18,19] we speculated that CnB1 could impact the onset of IMN via its effect on the T-cell activation mediated by calcineurin-NFAT pathway. Therefore, we manipulated CnB1 expression in Jurkat cells for further investigation. The results showed that downregulation of CnB1 inhibited the NFAT-regulated gene expression. However, no effect was observed when CnB1 was upregulated. It was likely because the calcineurin catalytic subunit was relatively insufficient in the cells. This explanation was supported by a report that CnB1 did not increase calcineurin catalytic subunit expression but protected it from degradation.[20]

The physiological roles of CnB1 in the immune system have been extensively studied. Previous research found that removal of CnB1 gene function led to impaired positive selection during thymocyte development.[21] Mice with CnB1-deficient B cells had reduced plasma cell differentiation and antigen-specific antibody production during immune responses, suggesting that CnB1 is essential for immunogenic B-cell responses.[22] These observations indicate that CnB1 may affect other immune processes besides T-cell activation in the development of IMN. To make it

clear, it is necessary to further explore the relationship between the CnB1 in the specific subpopulation of T or B cells and IMN.

Accumulating clinical trials have evaluated the efficacy and safety of CNIs in IMN patients,[23,24] showing the antiproteinuric effects of CNIs. However, the precise pharmacological mechanism how CNIs ameliorate proteinuria remains unclear. It has been reported that CNIs can directly protect kidney podocytes to reduce proteinuria.[25] On the basis of our study, the antiproteinuric role of CNIs may derive from their immunosuppressive effects.

In addition, we analyzed the potential associations of PBMCs CnB1 expression with the severity and clinical outcomes of IMN. Nevertheless, we did not find any significant relationship between PBMCs CnB1 expression and the clinical parameters of IMN patients before or after treatment. Studies with more cases are necessary for further exploration.

The present study indicated the potential role of CnB1 in the occurrence of IMN. The mechanism maybe involved the effect of CnB1 on the T-cell activation mediated by calcineurin-NFAT signaling. Despite the limitations including the lack of the study related to calcineurin catalytic subunit and the small number of enrolled patients, our findings provided a new insight into the role of CnB1 in the pathogenesis of IMN.

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