Original Articles/นิพนธ์ต้นฉบับ

Natural Regulatory T cells (nTreg) In Minimal Change Disease and IgM Nephropathy.

Louischaroen Y, MD.[1], Thongpan M[2], C.Chaiyaroj S[2], Keesukphan P, MD.[3], Chalermsanyakorn P, MD.[4], Sumethkul V[1], Kitiyakara C, MD.[1]

[1]Department of Medicine, [2]Faculty of Science, Mahidol University;
[3]Department of Family Medicine, [4]Department of Pathology,
Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand

Abstract

Background: IgM nephropathy is a variant of minimal change disease (MCD) and is the commonest cause of nephrotic syndrome among Thai adults. Although patients with IgM nephropathy generally respond well to treatment with corticosteroids, patients often have a remitting and relapsing course. The pathogenesis is not well understood. It has been proposed that MCD or IgM nephropathy reflects a disorder of T-lymphocytes. Recently, abnormalities in natural regulatory T cells (nTreg) have been implicated in a number of autoimmune diseases. This is a pilot study to examine the number of Foxp3+CD4+ T cells in patients with MCD or IgM nephropathy.

Methods: Eleven patients (2 patients with MCD and 9 patients with IgM nephropathy) and 25 normal control subjects were studied. T cell subpopulations were measured by flow cytometry.

Results: Overall, nTreg were identified by the presence of Foxp3+CD4+ T cells. There were no significant differences in number of CD3+, CD4+ T cells population and Foxp3+CD4+ T cells in MCD/IgM patients compared with normal subjects. In a subgroup analysis, there was a significant increase in Foxp3+CD4+ numbers in untreated MCD/IgM patients with active disease compared to controls.

Conclusion: These results suggesting correlation between activation of immune system in pathogenesis. Natural regulatory T cell as detected with Foxp3+CD4+ was increased in active MCD/IgM nephropathy patients.

Key words: Regulatory T cells; nTreg; Treg; T cells; FoxP3; minimal change disease; IgM nephropathy; nephrotic syndrome, kidney

Abbreviations: MCD = minimal change disease; IgM = IgM nephropathy; nTreg = natural regulatory T cells; FoxP3 = Forkhead box P3 transcription factor; CD = cluster of differentiation

Corresponding Author: Kitiyakara C, MD.
Renal Division, Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand Tel: 66-2-201-1400 Fax: 66-2-201-1400
E-mail: rackt@mahidol.ac.th

Introduction

Minimal change disease (MCD) describes a pathology appearance on light microscopy in which there are no definite changes from normal glomeruli. Clinically, MCD is characterized by nephrotic syndrome which generally responds well to treatment with corticosteroids[1]. World wide, MCD is the most common form of nephrotic syndrome in children, accounting for 90% of cases under the age 10 years and more than 50% in older children[2]. In the United States adults, MCD is the third most common form of nephrotic syndrome, after membranous nephropathy and focal segmental glomerulosclerosis (FSGS)[3].

IgM nephropathy is defined immunohistochemically by the presence of diffuse mesangial staining of glomeruli for IgM. These abnormalities are considered to be a variant of MCD[4,5]. IgM nephropathy is the most common cause of nephrotic syndrome in Thailand (45.2%)[6]. Several studies have shown similar clinical features in IgM nephropathy and MCD. In one study, both children with IgM nephropathy and MCD with nephrotic syndrome had 90% respond to prednisolone[7]. Both groups had similar relapse rates and good long term outcome.

The pathogenesis of MCD or IgM nephropathy is not well understood[1,8]. The responses of MCD to immunosuppressive drugs such as corticosteroids and cyclophosphamide implicate the involvement of immune system in the pathogenesis of MCD[9-11] or IgM nephropathy. It has been proposed that MCD reflects a disorder of T-lymphocytes, which may produce abnormal cytokines or permeability factors leading to increased glomerular permeability.

The regulatory T cells represent defined subpopulations of T cells with an important role in maintaining peripheral tolerance and the prevention of autoimmunity[12]. Various populations of nTeg have been described, including thymically derived CD4(+) CD25(+) natural regulatory T cells or nTreg. More recently, Forkhead box P3 transcription factor (FoxP3) Foxp3+ CD4+ T cells have been shown to be a more specific marker for nTreg than CD25+T cells since CD25+ is also expressed in activated T cells[13].

Foxp3+CD4+ (nTreg) cells have been shown essential roles in maintaining self-tolerance and modulating adaptive immune response. Thus abnormalities in the numbers or the function of nTreg has been documented in animal models of autoimmune diseases[14] and patients with autoimmune rheumatoid arthritis[15], type I diabetes mellitus[16], asthma[17-19], inflammatory bowel disease[20,21], autoimmune thyroiditis and myastinia gravis[22].

Abnormalities of nTreg may lead to abnormal T and B cells function contributing to the pathogenesis of T cell dysfunction observed in IgM or MCD. However, the number of nTreg has not been studied in this disease previously. The objectives for this study were to characterize the number of T cell subpopulations by flow cytometry especially nTreg by marker Foxp3+CD4+ transcription factor and other cluster of differentiation in patients with MCD/IgM nephropathy compared with those of normal subjects. T cell subpopulations and nTreg in subgroups of untreated patients and patients on treatment with corticosteroids will also be studied.

Materials and Methods

Healthy subjects

Normal control subjects were recruited from healthy volunteers. Subjects were screened for underlying diseases by history taking, physical examination, and blood tests. Blood and urine samples were sent to Ramathibodi Hospital to determine blood chemistry and clinical microscopy. The determined parameters were complete blood count, liver function tests, lipid profiles, blood glucose, blood urea nitrogen (BUN), creatinine, HBsAg, AntiHCV, antinuclear antibody and urinalysis. In addition to routine blood

sampling, ten ml of blood were drawn into a tube containing heparin for isolation of peripheral blood leukocytes.

Patient population

Patients undergoing kidney biopsy were evaluated by history taking, physical examination, and blood tests similar to normal subjects. Renal histopathology was evaluated by a single renal-specialist pathologist and was classified according to standard criteria. Patients with a biopsy-proven MCD or IgM were recruited.

Patients were classified as *active-untreated* if they had proteinuria and had never received corticosteroids or immunosuppressive drugs, *active on treatment* if they were receiving corticosteroids at the time of biopsy, *relapse on steroid* if they had achieved remission and relapsed while still being on corticosteroids. All patients were followed up for best response to corticosteroids, and they were classified as complete responder or partial responder. Complete response was defined as proteinuria 0 or trace or urine protein creatinine ratio <0.3 at anytime after treatment. Partial response was defined as proteinuria 1+ or 2+ or proteinuria <1 g/day or urine protein creatinine ratio <1 when proteinuria has been previously ≥3+ at anytime without achieving complete response on steroid.

This study was approved by the Ethical Committee at the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (ID 04-49-25). All subjects provided written informed consent.

Surface and intracellular staining of peripheral white blood cells

Surface staining was performed after incubating whole blood in red blood cell lysis buffer for 15 minutes. Cells were incubated with fluorochrome-labeled antibodies to CD3, CD4 (5 μg/ml, 106 cells/test). After incubation for 30 minutes at 4°C, they were washed twice with phosphate buffer saline (PBS) and fixed with 2% paraformaldehyde.

Intracellular staining was performed following the manufacturer's instruction (eBioscience, SanDiego, CA). Briefly, cells were stained for the surface markers and washed twice with PBS. Then, cell membrane was permeabilized with Fixation/Permeabilization Buffer (eBioscience, SanDiego, CA), at 4°C for 45 minutes in the dark. After washing twice with Permeabilization buffer, cells were stained with anti-human Foxp3 (15 μl/test) at 4°C for 30 minutes in the dark. Next, cells were washed twice with PBS and fixed with 2% paraformaldehyde. The samples were then used for flow cytometry analysis.

Flow cytometry analysis

Background fluorescence will be assessed using the appropriate isotype- and fluorochrome-matched control mAb to determine the percentage of positive cells. CD4+ T cells were gated on CD3+CD4+ cells using the relevant labeled antibodies. Percentage of Foxp3+CD4+ were based on CD4+ T cells. The analysis was performed using FACScanto™ (CellQuest™ software; Becton Dickinson). Percentage CD3+ cells were used to calculate total CD3+ cells from total CD3+ cells from total white count. Similarly total CD4+ cells were calculated from %CD4+ cells.

Reagents

Fluorescein isothiocyanate(FITC)-conjugated anti-FOXP3 (clone PCH101) mAb (eBioscience, SanDiego, CA), PE-Cy7 conjugated anti-CD3 (clone S4.1) mAb, PE conjugated anti-CD4 (clone S3.5) mAb (BD Bioscience, San Jose, CA) and also irrelevant isotype- and fluorochrome-matched and secondary control antibodies (BD Bioscience, San Jose, CA), were used in this study. Antibody concentrations used in cell staining are based on the data supplied by the manufacturers and initial optimization studies.

Statistical analysis

The normality of the distributions of all variable data in both control subjects and MCD/IgM patients were determined using the Komogorov-Smirnov goodness-of-fit test. Percentage levels of subtypes of T-cells population and mean fluorescence intensities of the cell staining were compared using the Student's t test for normally distributed populations and Mann-Whitney *U* test for non-normally distributed populations. The correlations between parameters were examined using calculation of the Pearson's correlation coefficient and Spearman's rank correlation test. All statistical analysis was performed using SPSS (SPSS, v11.5, SPSS Inc, Chicago, IL). *P* values <0.05 was considered significant.

Results

Patient population

Of 11 patients, two patients had MCD, and nine patients had IgM nephropathy. There were twenty-eight in normal subjects. Among them three were excluded because one had diabetes mellitus, one had positive HBsAg and one had high antinuclear antibody titer. Therefore twenty-five normal subjects were analyzed.

Table 1 shows the individual characteristics of patients with MCD/IgM nephropathy. Five patients were new untreated cases. Six subjects were on prednisolone at the time of blood sampling. Of subjects on treatment, two had new active disease, but had already initiated treatment with prednisolone prior to presentation to Ramathibodi, and three had relapses while on prednisolone (dose range 30-60 mg/day). In addition to prednisolone, one patient also received azathioprine.

Baseline characteristics of patients and controls are shown in Table 2. MCD/IgM nephropathy patients were older than controls. The sex ratios were similar. Median total white blood cell count and neutrophil count were higher than controls. Mean serum creatinine at baseline were similar in both groups and were in the normal range. Urine protein dipstick were negative in all normal subjects. Twenty-four hours urine protein in MCD/IgM patients was 3880±2808 mg. As expected, serum cholesterol was higher, and serum albumin was lower in patients with MCD/IgM.

T cells subpopulation and nTreg proportion

All patients

Figures 1 and 2 show that when all subjects were considered, CD3+ and CD4+ T lymphocytes were similar in MCD/IgM compared with normal subjects. Median (range) CD3+ number in normal subjects were 2309(865-4053)(/mm^3) and in MCD/IgM were 1226(519-5900)(/mm3), P = NS. CD4+ number in normal subjects were 1219(352-2451) (/mm^3) and in MCD/IgM were 1226(519-5900), P = NS. When both treated and untreated subjects were considered (Figure 3). Foxp3+CD4+or nTreg tended to be higher in patients than in controls although this did not reach statistical significance (Foxp3+CD4+/CD4+ 16.60(5.20-41.40)(%) in controls and 21.30(6.30-56.30)(%) in MCD/IgM (P = 0.064)).

Active-untreated patients

Figure 4 shows data in active-untreated group compared with normal subjects. There was a significant increase in Foxp3+CD4+ nTeg in the active-untreated group compared with normal subjects (23.80(16.30-30.00)(%) in active-untreated MCD/IgM VS 16.60(5.20-41.40)(%) in control; P = 0.026). The number of CD3+ and CD4+ T cells were similar between two groups. (CD3+ in active-untreated group 1527±1055 (/mm^3) VS control 2270±874(/mm^3); P = NS. CD4+ in active-untreated group 577(401-2295) (/mm^3) VS control 1217(352-2451) (/mm^3); P = NS.

nTreg and response to corticosteroids

As shown in Table1, complete response to cor-

Table 1 MCD/IgM nephropathy patients

No	Dx	Age (yr)	Sex	Prior disease duration (mo)	Activity of disease at blood sampling	Alb (g/dL)	Cr (mg/dL)	Cholesterol (mg/dL)	Uprot	24 hr Uprot (mg/day)	Dose of steroid at blood sampling (mg)	Response to treatment
1	MCD	57	F	1	active-untreated	8.5	0.9	588	4+	5678	60	partial response
2	MCD	30	F	1	active-untreated	47.0	0.5	403	4+	7638	0	refer*
3	IgM	79	M	3	active-untreated	35.9	2.0	138	2+	706	0	partial response
4	IgM	17	M	10	active on Rx°	8.9	0.7	644	4+	2543	30	complete response
5	IgM	60	M	36	relapse on steroid	31.8	0.9	LDL = 184	4+	ND†	30	complete response
6	IgM	52	F	19	relapse on steroid	32.0	0.5	398	0	1323	60	partial response
7	IgM	47	F	1	active on Rx°	25.9	0.6	276	4+	155	60	complete response
8	IgM	53	M	1	active-untreated	8.4	1.5	410	4+	7277	0	complete response
9	IgM	45	F	5	active-untreated	35.0	1.7	259	4+	4454	0	partial response
10	IgM	53	M	4	active-untreated	5.4	1.3	333	4+	ND†	0	refer*
11	IgM	33	F	54	relapse on steroid	34.8	0.6	225	2+	5150	50+ azathioprine 50 mg	complete response

†ND - no data available °Rx -treatment Alb- albumin, Cr- creatinine, Uprot-Urine protien

* refer - patient was refered to another hospital

Table 2 Baseline data in normal subjects and patients with MCD/IgM nephropathy

Variables	Group		MCD/P value
	Normal (N=25)	IgM (N=11)	
Age (years)	24 (20-42)	52 (17-79)	0.01
Sex			
Male	11 (44%)	5 (45.5%)	NS
Female	14(56%)	6 (54.5%)	NS
WBC (/mm^3)	5887 ± 1113	9443 ± 5062	0.04
Lymphocyte (%)	34.4 ± 7.2	27.2 ± 11.3	0.03
Neutrophil (%)	55.2 ± 8.1	64.1 ± 12.4	0.015
Absolute lymphocyte count(/mm^3)	1938 (1202-2916)	1920 (1065-5346)	NS
Absolute neutrophil count(/mm^3)	3122 (2107-5515)	4623 (2862-16153)	0.002
Serum albumin (g/L)	43.3 ± 3.2	24.9 ± 14.4	0.01
Serum creatinine (mg/dL)	0.9 ± 0.2	1.0 ± 0.5	NS
Serum cholesterol (mg/dL)	187.4 ± 28.8	367.4 ± 157.5	0.01

Data shown as mean±SD or median (min-max); NS: non significant

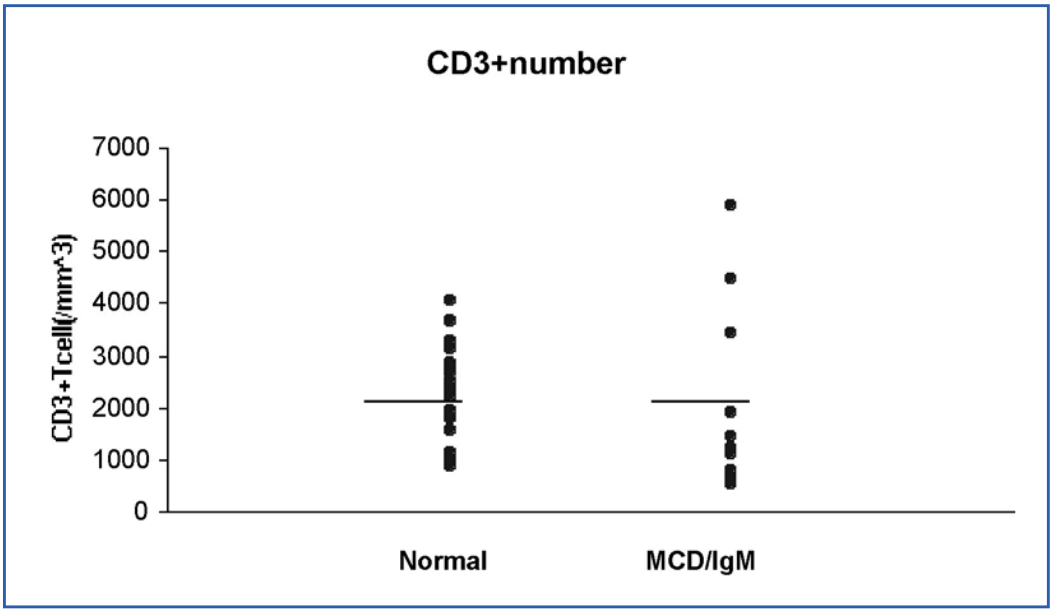

Figure 1 *CD3+ T cells number (/mm^3) in individual normal subjects (n=25) compared with individual MCD/IgM patients (n=11). P value = NS*

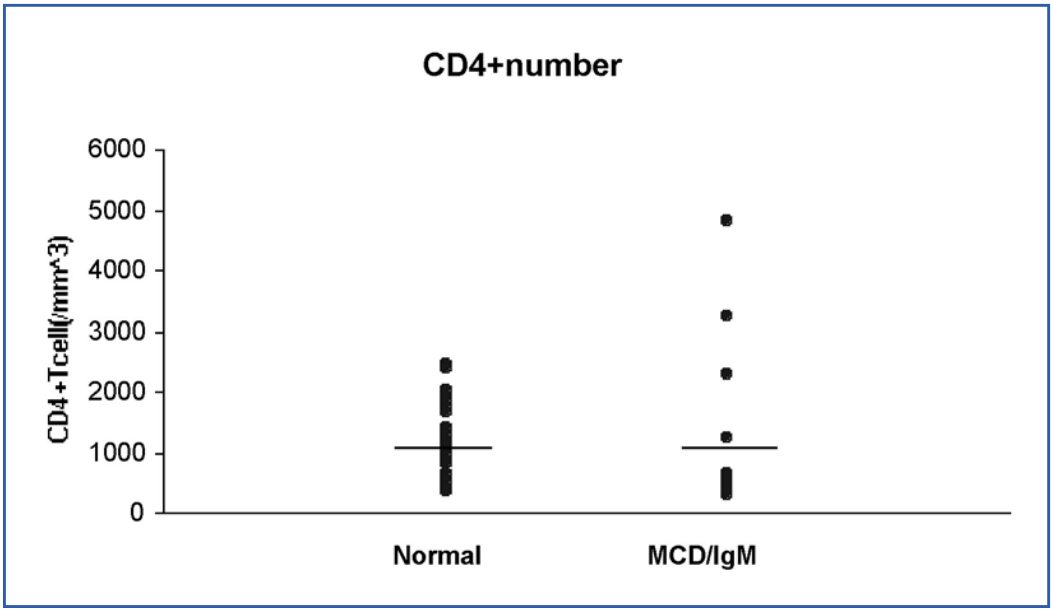

Figure 2 *CD4+ T cells (/mm^3) number in individual normal subjects (n=25) compared with individual MCD/IgM patients (n=11). P value = NS*

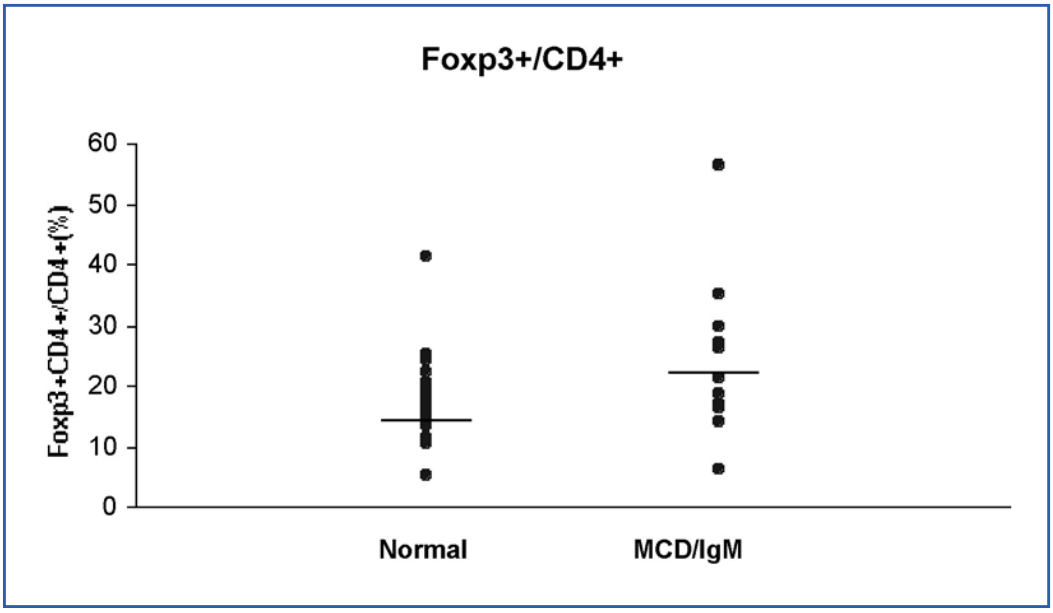

Figure 3 *Foxp3+CD4+/CD4+ (%) number in individual normal subjects (n=25) compared with individual MCD/IgM patients (n=11). P value = 0.064*

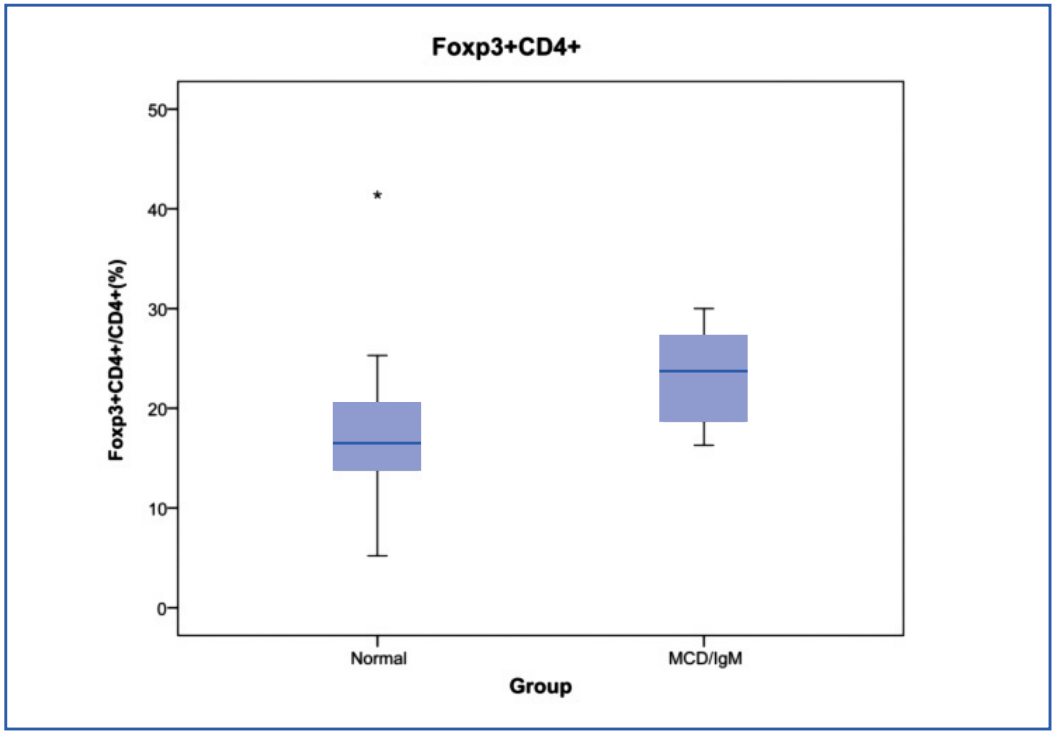

Figure 4 *Foxp3+CD4+ in normal subjects (n=25) compared with active-untreated MCD/IgM patients (n=5). *P <0.05*

ticosteroids was observed in 5 patients. Partial response was seen in 4 patients. Two patients were refered to another hospital and no data is available. There was no difference of Foxp3+CD4+ Tcells between complete and partial responder. Mean Foxp3+CD4+/CD4+ (%) in complete responder were 15.67±6.57 and in partial responder 29.98±16.91; P = NS.

Discussion

This study found that Foxp3+CD4+ nTreg were increased in untreated adult patients with active IgM or MCD compared to normal subjects. The total lymphocyte counts as evidenced from white count differentials or from CD3+ (Universal lymphocyte marker) were not different. Total T helper cell count as evidenced from CD4+ staining were also not different between patients and controls.

In MCD, a role for a pathogenic circulating factor leading to increased glomerular permeability has been proposed. Rats develop albuminuria upon injection of culture supernatants of stimulated peripheral blood mononuclear cells (PBMC) from patients with MCD[23-25]. T cells abnormalities have been implicated in the pathogenesis of MCD[26]. This is supported by the observation that MCD may develop after viral infection, in association with lymphomas or that the disease may respond to cyclosporine. Furthermore, a factor produced by human T-cell hybridomas derived from an MCD patient in relapse[27] produces albuminuria in rats. Thus circulating factors in MCD could be produced by abnormal T cells, but the mechanisms underlying the dysregulation T cells in MCD are not known. Several investigators believe that the pathogenic mechanisms underlying IgM nephropathy may be similar to MCD.

The lymphocyte subsets were examined in children with nephrotic syndrome due to MCD and normal children using immuno-alkaline phosphatase staining[28]. No significant differences were found in the proportion of CD3+, CD4+ T cells in children with MCD as compared to controls. These results are compatible with our study in adult patients with IgM or MCD since we also do not find changes in total CD3+ lymphocytes or the CD4+ population.

Natural T reg (nTreg) have been shown to regulate cognate immunity in autoimmune disease and allotransplantation[29]. Initially, nTreg were recognized by the expression of CD4+CD25+. However, CD25+ has been shown to be expressed also on activated T cells and here are not specific for nTreg. Recently, Foxp3+CD4+ has been shown to be a highly specific marker for nTreg[30,31]. The role of nTreg has not previously been studied in MCD or IgM with Foxp3+CD4+ as a marker.

Previously, one study found subpopulation of T cell CD4+CD25+ (IL-2 receptor positive) were significantly increased in comparison with normal healthy subjects[32]. Conversely, another study found that CD3+CD25+ cell were decreased in nephrotic syndrome patients[28]. Since both nTreg and activate T cells express CD25, it is not possible to distinguish which of these cells. Subpopulations are altered.

To date, no studies have examined the role of nTreg as defined by Foxp3+CD4+ in MCD. Foxp3+CD4+ is considered the most specific marker for nTreg[13]. Mutation of the Foxp3+CD4+ gene lead to severe autoimmune disease in human and mice. Decreased Foxp3+CD4+ numbers or function have been documented in experimental and human autoimmune diseases. When all patients are considered, the number of Foxp3+CD4+ are not significantly different between MCD and control subjects. Our patient group consisted of patients at different stages of renal disease. It is expected that corticosteroids therapy will alter the immune system and hence may alter the marker of Foxp3+CD4+ cells. Thus when we examined the subgroup of patients with active disease who have not yet received steroid, we found that the number of nTreg are increased in patients with MCD/IgM.

We had anticipated that nTreg would be decreased in MCD and thus contribute to poor control of autoimmune reactive T cells clones. The finding that nTreg is increased in untreated patient with active disease may imply that the function of nTreg is defective and the increase in nTreg number is compensatory for the defective function. Alternatively, nTreg may be increased as a secondary process in an attempt to control disturbance in other immune regulatory processes that are primarily defective. Previously, nTreg have been shown to be increased in patients with persistent hepatitis C virus (HCV) infection[33], Chagas disease[34] and nasopharyngeal carcinoma (NPC)[35].

These results demonstrated that pathogenesis of MCD and IgM nephropathy patients are related to immune dysregulation. T cell subpopulations such as nTreg as detected with Foxp3+CD4+ might be use as a markers for activity of these diseases, and to predict the good responsive to treatment. However, a limitation of this study was the small numbers of patients involved which might not represent all MCD and IgM nephropathy patients. In addition, the age differences between controls and diseased subjects may impact the results, This study documented the numbers of nTreg but did not assess their function. Further studies examining serial changes in nTreg should be performed in larger group of subjects with age-matched controls. Future understanding may lead to the development of rational targeted therapies of MCD/IgM in the future.

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Original Articles/นิพนธ์ต้นฉบับ

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ญาดา หลุยเจริญ, พบ.[1], มษฐา ทองปาน[2], ศันสนีย์ ไชยโรจน์[2], ประสิทธิ์ กี่สุขพันธ์, พบ.[3], พนัส เฉลิมแสนยากร, พบ.[4], วสันต์ สุเมธกุล[1], ม.ล.ชาดรีย์ กิติยากร, พบ.[1]

[1] ภาควิชาอายุรศาสตร์, [2] คณะวิทยาศาสตร์;
[3] ภาควิชาเวชศาสตร์ครอบครัว, [4] ภาควิชาพยาธิวิทยา,
คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล กรุงเทพฯ 10400

บทคัดย่อ

บทนำ: IgM nephropathy(IgM) เป็นโรคไตในกลุ่ม Minimal change disease (MCD) และเป็นสาเหตุของ nephrotic syndrome ในผู้ใหญ่ที่พบบ่อยที่สุดในประเทศไทย พยาธิกำเนิดของโรคนี้ยังไม่เป็นที่ทราบแน่ชัด มีสมมติฐานว่าอาจเกิดจากความผิดปกติของเม็ดเลือดขาวชนิด natural regulatory T cells (nTreg) ซึ่งมีบทบาทสำคัญในโรคที่เกี่ยวกับภูมิคุ้มกันต่อเนื้อเยื่อตนเอง

วัตถุประสงค์: เพื่อศึกษาปริมาณของเม็ดเลือดขาว nTreg ในผู้ป่วย MCD หรือ IgM nephropathy

วิธีการวิจัย: วัดจำนวน T cells โดยวิธี flow cytometry ในผู้ป่วย 11 คน (MCD 2 คนและ IgM 9 คน) และกลุ่มควบคุม 25 คน

ผลการศึกษา: การวัดจำนวน nTreg โดยการตรวจ Foxp3+CD4+ T cells พบว่าจำนวนของ CD3+, CD4+ และ Foxp3+CD4+ T cells ในผู้ป่วย MCD/IgM ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม การวิเคราะห์กลุ่มย่อยพบว่ามีการเพิ่มขึ้นของ Foxp3+CD4+ T cells อย่างมีนัยสำคัญทางสถิติในผู้ป่วย MCD/IgM ที่อยู่ในระยะกำเริบของโรคเทียบกับกลุ่มควบคุม และพบว่าผู้ป่วย MCD 1 คน มีการเปลี่ยนแปลงของจำนวน CD3+, CD4+ และ Foxp3+CD4+ T cells สัมพันธ์กับการตอบสนองในทางคลินิกต่อยากดภูมิคุ้มกันในกลุ่มสเตียรอยด์

การสรุปผลการศึกษา: การศึกษานี้สนับสนุนว่า พยาธิกำเนิดของโรคมีความสัมพันธ์กับระบบภูมิคุ้มกันในร่างกาย โดยพบว่าจำนวน nTreg ซึ่งวัดโดยการตรวจ Foxp3+CD4+ เพิ่มขึ้นในผู้ป่วย MCD/IgM ที่อยู่ในระยะกำเริบ