ORIGINAL ARTICLE

Bali Medical Journal (*Bali Med J*) 2018, Volume 7, Number 3: 639-644
P-ISSN.2089-1180, E-ISSN.2302-2914

Published by DiscoverSys

Effect of corticosteroid and vitamin D3 as combined therapy on 25 (OH) vitamin D serum level and regulatory T (TReg) cells population in children with idiopathic nephrotic syndrome

Krisni Subandiyah,* Harinda Khanifa, Astrid Kristina Kardani

ABSTRACT

Background: Nephrotic syndrome (NS) is a chronic disease in children that correlates with T lymphocyte dysregulation (imbalance of regulatory T cells (TReg)/T-helper 17 (Th17) cells ratio). Vitamin D3 serum level was known decreased in children with nephrotic syndrome. Low vitamin D3 serum level can affect the outcome of nephrotic syndrome management with corticosteroid therapy. This study was aimed to investigate the effect of vitamin D as adjuvant therapy to 25 (OH) Vitamin D serum level and TReg population in children with idiopathic nephrotic syndrome.

Method: This study was designed as a randomized clinical trial, double-blind, pre and post-test control group which involved 30 subjects that newly diagnosed as NS. Subjects were divided into two groups, prednisone and vitamin D treated group and prednisone only treated group. TReg population in peripheral blood mononuclear cells (PBMC) was analyzed using flow cytometry. Vitamin D serum level was measured by ELISA method.

Results: Results showed that there was a significant elevation of TReg (independent t-test, $p = 0.001$) and 25 (OH) vitamin D serum level (independent t-test, $p = 0.001$) in prednisone and vitamin D treated group as compared to prednisone only treated group. Pearson testing first group showed that vitamin D level was positively correlated with TReg ($p = 0.332$, $r = 0.183$). The number of early remissions was higher in group treated with steroid and vitamin D3 as a combined therapy compared to group treated with steroid only (50 % vs 20%). Whereas the number of late remission was higher in steroid only group (23% vs 6%).

Conclusions: We concluded that corticosteroid and vitamin D3 as a combined therapy increase both of TReg and 25 (OH) vitamin D level which affect glucocorticoid therapy response in subjects. The number of early remission was higher in group treated with corticosteroid and vitamin D3 as a combined therapy.

Keywords: 25 (OH) vitamin D, regulatory T (Treg) cells, corticosteroid, vitamin D3, nephrotic syndrome

Cite This Article: Subandiyah, K., Khanifa, H., Kardani, A.K. 2018. Effect of corticosteroid and vitamin D3 as combined therapy on 25 (OH) vitamin D serum level and regulatory T (TReg) cells population in children with idiopathic nephrotic syndrome. *Bali Medical Journal* 7(3): 639-644. DOI:10.15562/bmj.v7i3.769

Department of Child Health, Faculty of Medicine, Brawijaya University, Dr. Saiful Anwar Public Hospital, Malang, 65122 Indonesia

*Correspondence to:
Krisni Subandiyah, Department of Child Health, Faculty of Medicine, Brawijaya University, Dr. Saiful Anwar Public Hospital, Malang, 65122 Indonesia
krisdika2002@yahoo.com

Received: 2017-07-26
Accepted: 2018-6-25
Published: 2018-8-1

INTRODUCTION

Idiopathic nephrotic syndrome is the most glomerular disease which characterized by several clinical manifestations such as severe proteinuria, hypoalbuminemia, hyperlipidemia, and edema.[1,2] Prevalence of nephrotic syndrome was 12-16 cases per 100.000 children, and the peak was occurred at 2-5 years old. In Indonesia, the incidence of this disease was 6 cases per 100.000 children per year in children aged less than 14 years old and dominated by male (male : female ratio 2 : 1).[1,3]

Pathogenesis of nephrotic syndrome still based on immunological aberration which characterized by abundant circulating factor and immuno-regulatory imbalance.[4,5] Regulatory T-cells (TReg) is an important tolerogenic T-cells which possessed protective effect on podocyte destruction.[6,7]

Elevated vascular permeability and angiogenesis are pathologic manifestations of nephrotic syndrome which caused by IL-6-induced vascular endothelial growth factor (VEGF) production. IL-6 and transforming growth factor- β (TGF-β) would lead to differentiation of IL-17 secreting cells (Th17) and inhibit TGF-β-induced TReg differentiation, suggesting that IL-16 played an important role in TReg/Th17 equilibrium.[8] Vitamin D had been known as immune-modulator and possessed pleiotropic effects such as induction of TReg differentiation,[9,10] inhibition of Th1 and Th17 effector cells,[9,11] induction of immature and tolerogenic dendritic cells,[12] inhibition of memory B cell formation and plasma cell differentiation.[9,13]

This study was aimed to investigate the effect of vitamin D as adjuvant therapy to 25 (OH) Vitamin D level and Treg cells population in pediatric nephrotic syndrome.

METHODS

Study Design

This study was designed as a randomized clinical trial (RCT) double blind, pre and post-test control group. There were two groups namely G1 (prednisone and vitamin D3) and G2 (prednisone only). TReg, and vitamin D level were measured before and after treatment. Treatment for G1 was prednisone 2 mg/kg body weight/day (maximal dose 80 mg/day) and vitamin D3 oral preparation 2000 IU (D-Vit, PT Gracia PharmindoTM) 2000 IU/day for four weeks. Treatment for G2 was prednisone 2 mg/kg body weight/day (maximal dose 80 mg/day) for four weeks according to ISKDC protocol. All the procedure and treatment of this study had been approved by Ethical Committee Faculty of Medicine, Brawijaya University, Malang.

SUBJECTS

As many as 30 subjects were included in this study (15 subjects G1, 15 subjects G2). Subjects were taken from Pediatric Nephrology Outpatient Care and Pediatric Ward, dr. Saiful Anwar Public Hospital, Malang during January - December 2016. Inclusion criteria for this study were the newly diagnosed nephrotic syndrome, age 1-14 years old, and allowed by his/her parents (informed consent). Exclusion criteria for this study were the secondary nephrotic syndrome, congenital nephrotic syndrome, relapse nephrotic syndrome, and steroid dependent nephrotic syndrome.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Blood sample in EDTA vacutainer was mixed until homogeneous before added PBS 1:1. Blood sample-PBS mixture was taken by using micropipette and transferred slowly into falcon tube wall that already filled with Ficoll-Hipaque d=1.077 g/dl (blood sample-PBS mixture volume: Ficoll-Hipaque volume was 1:1). This mixture was centrifuged at 1500 rpm, room temperature, for 30 minutes and would be resulted in the formation of 4 layers that was plasma, PBMC, Ficoll-Hipaque and erythrocyte. PBMC ring was taken slowly using micropipette and transferred into 15 mL centrifuge bottle. Washed with 10 mL PBS and centrifuged at 1200 rpm, room temperature for 10 minutes. The supernatant was removed, washed using PBS, and centrifuged again at 1200 rpm, room temperature, for 10 minutes. After two times washing and centrifugation process, PBMC formed as a pellet in the bottom of centrifuge bottle.

Measurement of regulatory T-cell population

Measurement of TReg was based on flow cytometry. In this experiment, we used phycoerythrin (PE) anti-human FOXP3, FITC anti-human CD4 and PE/Cy5 anti-human CD25 (eBioscience, San Diego, CA). PBMC was suspended at a certain density (2 × 106 cells/ml) in culture medium (RPMI which equipped with penicillin 100 U/ml, streptomycin 100µg/ml, glutamine 2mM, 10% calf fetal serum). The cell suspension was transferred into 24 wells. Cell culture then stimulated with phorbol myristate acetate (PMA) 50 ng/ml and ionomycin 1µM for 4 hours in monensin 500ng/ml (Alexis Biochemical, San Diego, CA). Incubator temperature set at 37°C and 5% CO2. After 4 hours culture, cell culture was transferred to a sterile tube and centrifuged at 1500 rpm for 15 minutes.

T-cell lymphocyte was transferred into a new tube, washed with phosphate-buffered saline (PBS), and then incubated with fluorescein isothiocyanate (FITC) anti-human CD4 and PE CD25 at temperature 4°C for 30 minutes. After incubation, specimens were stained with PE anti-human Foxp3. Specimens were transferred into cuvette ready for flow cytometry analysis. TReg population was analyzed with BD Cell Quest Pro.

Measurements of Vitamin D Level

Measurement of vitamin D level was based on ELISA method as previously described. Briefly, 200µl prediluted serum samples were added to each well and then incubated for 2 hours at 25°C. After washing process, 100 µl enzyme conjugate was added and incubated for 30 minutes at room temperature. After washing process, 100µl chromogen/ substrates solution was added and incubated for 15 minutes at room temperature and dark room. Finally, 100µl stop solution was added to each well. After 30 minutes, specimens were ready for analysis using ELISA reader at 650 nm.

Statistical analysis

The statistical test was based on data distribution and homogeneity. Statistical differences of TReg, and vitamin D level between groups were analyzed by independent t-test. Correlation of TReg and vitamin D level was analyzed by Pearson correlation test. Data were analyzed at 95% confidence interval (α=0,05) using SPSS for Windows version 19.0.

RESULTS

Subject Characteristics

The subject characteristics such as age, sex, vitamin D status, outcomes (steroid sensitive or resistant),

Table 1 Baseline Characteristic

Sample Characteristic	Prednisone + Vit D (n = 15)	Prednisone (n = 15)
Age (years):		
1-≤ 5 years old	6 (6/30)	0
>5 -<10 years old	11 (11/30)	10 (10/30)
≥ 10-14 years	0	3 (3/30)
Sex:		
Boys	12 (12/30)	11(11/30)
Girl	3 (3/30)	4 (4/30)
Vitamin D status :		
Normal	0	9 (9/30)
Insufficiency	11 (11/30)	8 (8/30)
Deficiency	1 (1/30)	1 (1/30)
Nutritional status:		
Well nourished	13 (10/30)	13 (11/30)
Undernourished	2 (2/30)	2 (2/30)

Table 2 Clinical Outcome

Outcome	Prednisone + Vit D (n = 15)	Prednisone (n = 15)
Classification		
SSNS	15 (15/30)	13 (13/30)
RSNS	0	2 (2/30)
Remission		
Early	13 (13/30)	6 (6/30)
Late	2 (2/30)	7 (7/30)
No remission	0	2 (2/30)

Table 3 TReg Percentage Before Treatment, After Treatment, and Its Enhancement After Treatment

		Treatment		
		Prednisone + Vit D	Prednisone	
Variable	Group	Mean ±SD	Mean ± SD	P-value
Treg Percentage (%)	Pretest	3.44 ± 2.55	3.66 ± 1.80	0.791
	Posttest	17.27 ± 6.7	7.85 ± 7.52	0.001
	Deviation	13.82 ± 6.04	4.19 ± 7.40	0.001

Table 4 Vitamin D level before treatment, after treatment, and its elevation after treatment.

		Treatment		
		Prednisone + Vit D	Prednisone	
Variable	Group	Mean ±SD	Mean ± SD	P-value
Vit D level ng/mL	Pretest	14.87 ± 7.47	27.25 ± 14.99	0.008
	Posttest	46.34 ± 14.19	40.66 ± 13.96	0.281
	Deviation	31.46 ± 9.69	13.43 ± 17.25	0.001

Table 5 **Correlation of TRegand Vitamin D Level**

Variable correlation	n	Correlation Coefficient (r)	p-value
Treg elevation associated with elevation of Vit D level	30	0.183	0.332 >

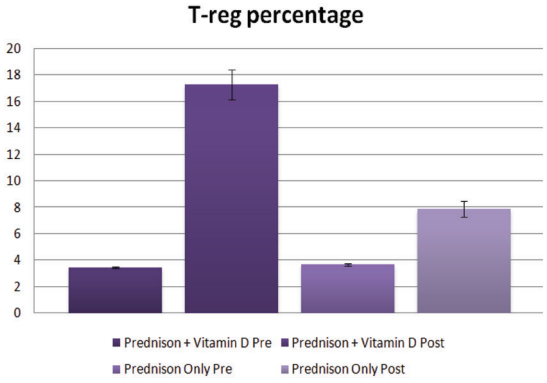

Figure 1 Treg percentage before (pretest) and after (post test) treatment. Treg mean percentage in G1 (3.44 ± 2.55%) and G2 (3.66 ± 1.80%) on pretest evaluation and Treg mean percentage in G1 (17.27 ± 6.7%) and G2 (7.85 ±7.52%) on post test evaluation

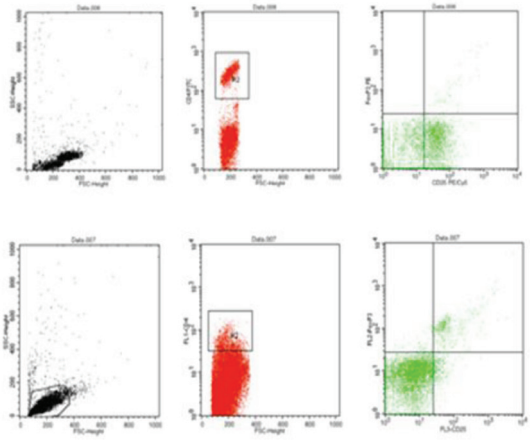

Figure 2 TReg percentation on new nephrotic syndrome before and after prednisone treatment. Above: R2 are PE stained CD4 cell before treatment. The upper right figure is CD4CD25+Foxp3+ percentation which stained using FITC before treatment. Below: R2 is PE stained CD4 cell after treatment. The lower right figure is CD4CD25+Foxp3+ percentation which stained using FITC after treatment

and nutritional status were shown in Table 1. Clinical outcomes of subjects were shown in Table 2. Remission before four weeks and remission after

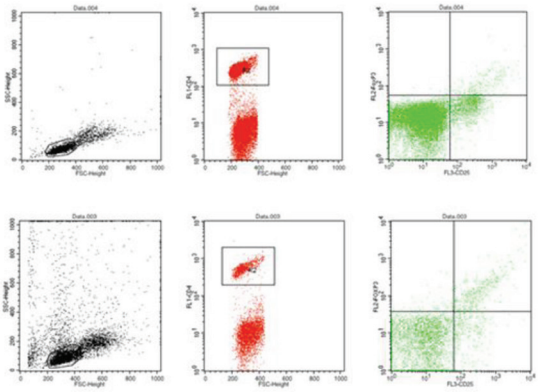

Figure 3 TReg percentation on new nephrotic syndrome before and after prednisone and vitamin D treatment. Above: R2 are PE stained CD4 cell before treatment. The upper right figure is CD4CD25+Foxp3+ percentation which stained using FITC before treatment. Below: R2 is PE stained CD4 cell after treatment. The lower right figure is CD4CD25+Foxp3+ percentation which stained using FITC after treatment

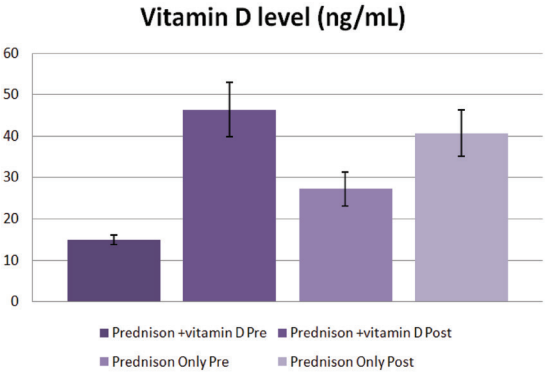

Figure 4 Vitamin D level before treatment, after treatment, and its elevation after treatment. Vitamin D level in G1 (14.87 ±7.47) and G2 (27.25 ± 14.99) on pretest evaluation and Vitamin D level in G1 (46.34 ± 14.19) and G2 (40.66 ± 13.96) on post test evaluation

four weeks were found in both of group but earlier in G1. Most of subjects were steroid sensitive nephrotic syndrome (SSNS), only two patients did not get remission and classified as steroid resistant nephrotic syndrome (SRNS).

Published by DiscoverSys | *Bali Med J* 2018; 7(3): 639-644 | doi: 10.15562/bmj.v7i3.769

Regulatory T cell population

The result showed that the degree of elevation Treg was higher in G1 compared to G2 (before and after treatment, independent t-test, p = 0.001). Table 3 and Figure 1-3 showed TReg percentage before treatment, after treatment, and its enhancement after treatment.

Vitamin D level

The result showed that the degree of elevation vitamin D was higher in G1 compared to G2 (before and after treatment, independent t-test, p = 0.001). Table 4 and Figure 4 showed vitamin D level before treatment, after treatment, and its elevation after treatment.

Correlation of TReg and vitamin D level

The result showed that TReg percentage was positively correlated with vitamin D level (p = 0.332, r = 0.183).

DISCUSSION

Baseline Characteristics

Based on the age factor, data showed that the most age distribution of subjects was less than 10 years old. 30 subjects included in this study, 23 was a male. This finding was also in accordance with the previous study and had been considered to be correlated with abnormal T cells clone in male thymus gland.[14] Nutritional status revealed that most subjects were in good nutrition. However, it is important to evaluate the nutritional status of children with nephrotic syndrome because they were at high risk to be suffered from malnourished.

Vitamin D status in nephrotic syndrome patients (21 of 30) was at a low level. This result was accordance with the previous study conducted in General Hospital dr. Cipto Mangunkusumo which had been reported that 22 of 26 nephrotic patients had low vitamin D level (10 insufficiency, 16 deficiency).[15] Loss of vitamin D-bounded protein through urine had been considered as an etiologic factor of its low plasma concentration in nephrotic patient.[16] Low vitamin D level caused hyper-reactivity dendritic cell, Tcell and Bcell; TReg suppression and elevated pro-inflammatory cytokines17 which would lead to nephrotic syndrome. Several factors which affect 25(OH)D level such as age, race, season, and milk consumption.[18]

Vitamin D and TReg Population

There was significant elevation TReg in prednisone and vitamin D treated group was compared to prednisone only treated group. Reduction of TReg count and its dysfunction in nephrotic syndrome would lead to inability to suppress effector T cells19and associated with degree of proteinuria.[20,21] TReg was also act as anti-inflammatory T cells through secretion of several anti-inflammatory cytokines such as IL-10 and TGF-β.[22]

Vitamin D administration could induce and stimulate TReg directly through antigen presenting cells or dendritic cells and indirectly through endocrine or intracrine conversion 25(OH)D became 1,25(OH)2D3.[22] Furthermore, vitamin D administration was correlated with elevation of TReg Foxp3+ count.[10,11,23] Several mechanisms which focused on how vitamin D affects TReg had been studied. Administration of 1,25(OH)2D3 could enhance STAT5 phosphorylation in Foxp3+ cells via TGF-β and IL-2 stimulation which in turn would lead to TReg differentiation.[24,25] Conversely, low vitamin D level would lead to IL-6 upregulation instead of TGF-β downregulation which in turn could cause Th17 differentiation.[25]

Glucocorticoid had been known as anti-inflammation drugs which induce T cell apoptosis, induce T cell anergy, and suppress T cell function.[26] Furthermore, glucocorticoid could induce IL-10 upregulation in immature dendritic cells or macrophage thus induce differentiation of TReg/ suppressor T cells.[26] Adjuvant therapy vitamin D3 could potentiate immunosuppressive effects of TReg through upregulation of Foxp3 and IL-10.[10,27,28]

Correlation of TReg and Vitamin D Level

This study had been proved that vitamin D have positively correlated with TReg. Zhou and colleagues (2008) had been reported that Th17 cytokines level such as IL-17 and IL-23 were elevated in nephrotic patients and this condition was related to glomerular destruction, proteinuria, and hypoalbuminemia. Furthermore, imbalance of Th17/TReg population which represents pro-inflammatory condition occurred in patients with minimal change nephrotic syndrome.30 Our data are suggesting that vitamin D played an important role in the regulation of TReg cells.

Limitation of Study

Several factors as follow could affect the result of study such as less subject number, other factors which could affect vitamin D level (nutrition intake such as milk, sun exposure), other biomolecular factors that didn't studied such as IL-23, vitamin D receptor, and CD80.

CONCLUSION

We concluded that there was a significant elevation of Vit D serum level and TReg in prednisone and vitamin D treated group as compared to prednisone only treated group. The number of early remission

was higher in group treated with corticosteroid and vitamin D3 as a combined therapy.

CONFLICT OF INTEREST

All authors declare there is no conflict of interest regarding publication of this manuscript.

ACKNOWLEDGEMENTS

This research was partially supported by Frisian Flag Indonesia. We thank our colleagues from central committee of Indonesian Pediatric Society for contributing this research.

REFERENCES

1. Bagga A. Management of steroid sensitive nephrotic: revised guidelines. Indian Pediatr2008;45: 203-14.
2. Zhang S, Audard V, Fan Q, Pawlak A, Lang P, Sahali L. Immunopathogenesis of idiopathic nephrotic syndrome. Contributions to Nephrology2011;169: 94-106.
3. UKK Nefrologi Ikatan Dokter Anak Indonesia. Tatalaksana sindrom nefrotik idiopatik pada anak. Jakarta: Badan Penerbit Ikatan Dokter Anak Indonesia; 2008.
4. Hafez MA, Shimada M, Lee PY, Johnson RJ, Garin EH. Idiopathic nephrotic syndrome and atopy: is there a common link? American Journal of Kidney Disease 2009;54(5): 945-53.
5. Wang L, Li Q, Li C, Yang H, Wang X, Tao H.The role of Th17/IL-17 in the pathogenesis of primary nephrotic syndrome in children. Kidney Blood Press Res 2013;37: 332-345.
6. Wang.Regulatory T cells in renal disease. Int J Clin Exp Med2008;1: 294-304.
7. Pereira WF. The role of the immune system in idiopathic nephrotic syndrome: a review of clinical and experimental studies. Inflamm Res 2013; 2: 11-16.
8. Handono K, Hasanah D, Kalim H, Mawarti H.The associations among serum levels of vitamin D,TGF-β/IL-6 balance and Treg/Th17 balance in systemic lupus erythematosus patients in Indonesia. IntJBiochemBiotechnol2013;2(9): 490-496.
9. Terrier B, Derian N, Schoindre Y, Chaara W, Geri G, Zahr N, et a. Restoration of regulatory and effector T cell balance and B cell homeostasis in systemic lupus erythematosus patients through vitamin D supplementation. Arthritis Research & Therapy2012;14: 1-10.
10. Urry Z, Chambers ES, Xystrakis R, Dimeloe S,Richards DF, Sov´a LG, et al. The role of 1α,25 dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3+and IL-10+ CD4+ T cells. Eur J Immunol2012;42: 2697–2708.
11. Prietl B, Pilz S, Wolf M, Tomaschitz A, Obermayer-Pietsch B, Graninger W, et al.Vitamin D supplementation and regulatory T cells in apparently healthy subjects: vitamin D treatment for autoimmune disease?IMAJ2010;12: 11-16.
12. Penna G, Amuchastegui S, Giarratana N, Daniel KC, Vulcano M, Sozzani S.1,25-Dihyroxyvitamin D3 selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. J Immunol2007; 178: 145-53.
13. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. J Immunol 2007;179: 1634-47.
14. Berg JG, Weening JJ. Role of the immune system in pathogenesis of idiopathic nephrotic syndrome. Clinical Science 2004;107: 125-36.
15. SeptariniAD, Tambunan T, Amalia P. Calcium and vitamin D supplementation in children with frequently relapsing and steroid-dependent nephrotic syndrome. Paediatr Indones2012;52: 1.
16. Esmaeeili M, Azarfar A, Hoseinalizadeh S.Calcium and vitamin D metabolism in pediatric nephrotic syndrome: an update on the existing literature.Int J Pediatr2015;15(3): 2-1.
17. Ginanjar E, Sumariyono, Setiati S, Setiyohadi B.Vitamin D and autoimmune disease. Acta med Indones-Indones J Intern Med 2007;39(3): 133-41.
18. Weng FL, Schults J, Heskovitz RM, Zenel BS, Leonard MB. Vitamin D insufficiency in steroid-sensitive nephrotic syndrome in remission. Pediatr Nephrol2005;20: 56-63.
19. Araya C, Diaz L, Wasserfall C, Atkinson M, Mu W, Johnson R, Garin E. T regulatory cell function in idiopathic minimal lesion nephrotic syndrome. Pediatr Nephrol2009;24(9): 1691-8.
20. Liu L, Qin Y, Cai J, Wang H, Tao J, Li H, et al. Th17/Treg imbalance in adult patients with minimal change nephrotic syndrome. Clinical Immunology2011;139: 314-20.
21. Shao XS, Yang XQ, Zhao XD, Liu Q, Xie YY, Wang XG, et al. The prevalence of Th17 cells and FOXP3 regulatory T cells (Treg) in children with primary nephrotic syndrome. Pediatr Nephrol2009;24:1683-1690.
22. Lang CL, Wang MH, Chiang CK, Lu KC.Vitamin D and the Immune system from the nephrologist's viewpoint. ISRN Endocrinology2014;14:1-11.
23. Smolders J, Thewissen M, Peelen E, Menheere P, Cohen Tervaert JW, Damoiseaux J. Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis. PLoS ONE2009;4(8): 63-45.
24. ChambersES, Suwannasaen D, Mann EH, Urry Z,Richards DF, Lertmemonkolchai G, et al. 1,25-dihydroxyvitamin D3 in combination with transforming growth factor-β increases the frequency of Foxp3+ regulatory T cells through preferential expansion and usage of interleukin-2. Immunology2014;143: 52-60.
25. Einstein R, Gordillo R, Spitzer A.The nephrotic syndrome. Pediatr Rev2009;30(3):94-104.
26. Franchimont D. Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. Ann NY Acad Sci2004;1024:124-37.
27. Heine G, Niesner U, Chang H, Steinmeyer A, Zugel U, Zuberbier T, et al. 1,25-dihydroxyvitamin D3 promotes IL-10 production in human B cells. Eur JImmunol 2008;38: 2210-8.
28. Barrat FJ, Cua DJ, Boonstra A,Richards DF, Crain C, Savelkol HF, et al. In vitro generation of interleukin-10-producing regulatory CD4+ cells is induced by immunosuppressive drugs and inhibited by T helper type 1(Th1)- and Th2-inducing cytokines. JExp Med2002;195: 603-16.
29. Kurts C, Panzer U, Anders H, Rees AJ.The immune system and kidney disease: basic concepts and clinical implications. Nature Reviews Immunology2013;13: 738-753.
30. Zhou L, Lopes JE, Chong MM, Ivanol Il, Min R, Victora GD.TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing ROR-gamma-t function. Nature2008;453(7192): 236-40.