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Vitamin K₂ immunosuppressive effect on pediatric patients with atopic dermatitis

Running title: Immunosuppressive effects of vitamin K₂

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Abstract

Backgrounds: Over 20 kinds of steroid, tacrolimus ointments and cyclosporine capsule are usually recommended for the treatment of atopic dermatitis (AD) according to the symptoms of patients. However, several side effects sometimes disturb the extensive use of these agents for the treatment of pediatric AD patients. The purpose of this study was to explore whether vitamin K₂ could be a new immunosuppressive candidate for pediatric patients with AD.

Methods: Immunosuppressive efficacies of vitamin K₂ were evaluated through a cell-culture procedures using mitogen-activated peripheral blood mononuclear cells (PBMCs) obtained from pediatric AD patients.

Results: The mean (SD) IC₅₀ values of vitamin K₂ on the proliferation of concanavalin A-activated PBMCs was 15.37 (30.05) μ M, while those of tacrolimus was 0.10 (0.28) ng/mL (0.12 (0.35) nM). There was a significant correlation between the IC₅₀ values of vitamin K₂ and those of tacrolimus ($P=0.0001$, $r=0.8871$). Whereas, there was no significant correlation between the IC₅₀ values of vitamin K₂ and those of cyclosporine A or methylprednisolone. A significant correlation between the IC₅₀ values of vitamin K₂ or tacrolimus and the blood eosinophil counts ($P=0.0099$, $r=0.7086$ and $P=0.0032$, $r=0.7722$, respectively) was observed.

Conclusion: Vitamin K₂ inhibited T cell mitogen-stimulated proliferation of PBMCs from pediatric AD patients dose-dependently. PBMCs from pediatric AD patients were more sensitive to the immunosuppressive efficacy of vitamin K₂, comparing with the PBMCs from healthy subjects. The individual immunosuppressive pharmacological efficacies of vitamin K₂ and tacrolimus could be inferred from the blood eosinophil count of the pediatric AD patients.

Keywords: immunosuppressive efficacy; pediatric atopic dermatitis; peripheral blood mononuclear cells; vitamin K₂

1. Introduction

Atopic dermatitis (AD) is clinically characterized by chronic, pruritic eczematous skin lesions and is accompanied by elevated serum concentrations of immunoglobulin E ¹. Aberrations in immune regulatory system is considered to be related to the pathogenesis of the disease ². It has been reported that 10 to 20% of children and teenagers were affected by AD in Europe ³, while the prevalence in Japan was 11.2% ⁴. Immunosuppressive drugs including more than 20 kinds of steroids or tacrolimus ointments are usually recommended for treatment of these patients based on the appropriate diagnosis and evaluation of cutaneous symptoms ⁵. As a categorized calcineurin inhibitor, tacrolimus ointment is the first choice for treatment of AD patients ⁶. Oral cyclosporine is another adaptive for adult AD patients in Japan. However, the side effects of tacrolimus ointment and cyclosporine capsule were recorded in 70% of the patients ⁷. The risk-benefit balance of oral steroids has also been difficult to evaluate because of the systemic adverse effects, especially in children, and the rebound phenomenon following treatment cessation ⁸. Thus, it has been a dilemma for pediatricians to choose the suitable immunosuppressant for AD patients ⁹.

Vitamin K₂, also known as menaquinone-4, administered at a pharmacological dosage of 45 mg/day, has been used for the treatment of osteoporosis in Japan ¹⁰. Our previous work demonstrated that vitamin K₂ suppressed the proliferation of T-cell mitogen-activated peripheral blood mononuclear cells (PBMCs) of healthy subjects *in vitro* ⁹. Moreover, vitamin K₂ reduced the percentage of CD4⁺ and CD4⁺CD25⁺ cells in the activated PBMCs of pediatric AD patients, as well as the amount of IL-2 produced from the cells. These results suggested that vitamin K₂ could modulate T-cell function in PBMCs of pediatric AD patients ⁹.

In the present study, we evaluated the immunosuppressive properties of vitamin K₂ on the proliferation of T-cell mitogen-activated PBMCs obtained from pediatric AD patients, and related the drug efficacies with the clinical characteristics or laboratory data of the patients. To further characterize the clinical application of vitamin K₂ to AD, we also compared the pharmacological

efficacies of vitamin K₂ on the activated PBMCs of pediatric AD patients with those of cyclosporine A, tacrolimus, and methylprednisolone.

2. Materials and Methods

2.1. Reagents

RPMI1640 and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). Concanavalin A was purchased from Seikagaku Kogyo Co., Tokyo, Japan. Cyclosporine A was obtained by Wako Chemicals, Co. (Tokyo, Japan). Tacrolimus was obtained by Toront research chemicals Inc. (Toront, Canada). Methylprednisolone and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Aldrich (St. Louis, MO, U.S.A.). Vitamin K₂ was provided by Wako Chemicals, Co. (Tokyo, Japan). Lymphocyte separation solution was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of the highest quality available from commercial vendors.

2.2. Subjects

The present study was carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki)*. The study was approved by the institutional ethics committee for studies in human in both Tokyo Medical University Hospital and the Tokyo University of Pharmacy and Life Sciences. Total of 12 AD patients and 12 healthy subjects were enrolled in this study. All pediatric atopic dermatitis patients (two females and ten males with the age of 6.0±3.8 years) and healthy subjects (six female and six males with the age of 26.8±2.2 years) gave their written informed consent for blood donation. Healthy subjects had neither a history of immunological disorders nor a history of taking immunosuppressive drugs. Basic characteristics of the patients including gender, age, scoring atopic dermatitis (SCORAD), blood eosinophil count, serum total IgE, specific IgE in serum (*Dermatophagoides pteronissinus*), thymus activation-regulated chemokine (TARC) and stimulation index were shown in **Table 1**.

2.3. Isolation of PBMCs and PBMC culture

Twenty milliliters of heparinized venous blood were taken from healthy subjects and patients. PBMCs were separated and suspended with RPMI 1640 medium containing 10% fetal bovine serum, 100,000IU/L penicillin and 100mg/L streptomycin to a final density of 1×10^6 cells/mL as we described previously¹¹⁻¹³. 196 μ L of the PBMC suspension, as prepared above, were placed into each well of non-treated 96-well microplate. Concanavalin A, as a T-cell mitogen, was added to each well to a final concentration of 5.0 μ g/mL. Subsequently, 4 μ L of ethanol solution containing methylprednisolone or cyclosporine A were subsequently added to give a serial concentrations of 0.01, 0.1, 1, 10, 100, 1000, and 10000 ng/mL, respectively. In case of tacrolimus, 4 μ L of ethanol solution were added to give final concentrations of 0.01, 0.1, 1, 10, 100 and 1000ng/mL. For vitamin K₂, 4 μ L of ethanol solution were added to give final concentrations of 0.1, 1, 10, and 100 μ M. The same volume of ethanol was added to the control wells (vehicle group). The plate was incubated for 96 h in an atmosphere of 5 % CO₂ at 37°C.

2.4. Evaluation of immunosuppressant pharmacological efficacy with MTT assay

After 96h of culture, 10 μ L of 5mg/mL MTT solution dissolved in saline was added to each well, and then the cultures were re-incubated under 5 % CO₂ at 37°C for 4 h¹⁴⁻¹⁶. The plates were centrifuged at 375 $\times$ g for 5min to precipitate cells and formazan produced by growing cells. Aliquots of the supernatant were removed from each well and dimethyl sulfoxide was added followed. The absorbance was read with a microplate reader at 550nm. Dose-response curves were plotted, and the IC₅₀ values of each immunosuppressive drug were calculated.

2.5. Statistical analysis

Differences of the MTT values for PBMC proliferation between the pediatric AD patients and healthy subjects were performed using Mann-Whitney test. The correlation between the IC₅₀ values of immunosuppressive drugs and the basic characteristics of pediatric AD patients was analyzed using Pearson's test. Differences of median values for the drug IC₅₀ or stimulation indices between any two subject groups were analyzed by Mann-Whitney's U-tests. The MTT

values in PBMCs cultured in the presence of vitamin K₂ were compared with those in PBMCs cultured in the absence of drug (control), and the differences were analyzed by Dunnett's multiple comparison test. These analyses were performed with GraphPad PRISM 5.0 (GraphPad Software Inc., San Diego, CA). In each case, two-sided *P* values <0.05 were considered to be significant.

3. Results

3.1. *Effects of cyclosporine A, tacrolimus and methylprednisolone on the proliferation of mitogen-activated PBMCs of pediatric AD patients and healthy subjects*

Typical dose-response curves of cyclosporine A, tacrolimus and methylprednisolone on the PBMC proliferation were presented in **Figure 1a**. The mean (SD) values obtained with the MTT assay in PBMCs cultured in the presence of these drugs, as well as concanavalin A, were shown in **Figure 1b, 1c and 1d**. The mean (SD) of IC₅₀ values of cyclosporine A, tacrolimus and methylprednisolone in PBMCs of pediatric AD patients were 2.00 (1.19), 0.10 (0.28), and 2.75 (3.83) ng/mL, respectively, whereas the mean (SD) IC₅₀ values of these immunosuppressive drugs in PBMCs of healthy subjects were 206.40 (408.19), 0.19 (0.27), and 42.21 (57.20) ng/mL, respectively (**Table 2**). The IC₅₀ values of cyclosporine A (*P*=0.0005) and methylprednisolone (*P*=0.0024) in PBMCs of pediatric AD patients were significantly lower than those in PBMCs of healthy subjects (**Figure 2**).

3.2. *Effects of vitamin K₂ on mitogen-activated proliferation of PBMCs of pediatric AD patients and healthy subjects*

Effects of vitamin K₂ against the concanavalin A-stimulated proliferation of PBMCs of the AD patients and the healthy subjects were presented in **Figure 3**. Vitamin K₂ significantly suppressed the PBMC proliferation at 10 μM. Vitamin K₂ at 100 μM showed the strongest anti-proliferative effect on the PBMC proliferation in both pediatric AD patients and healthy subjects (**Figure 3a**) (*P*<0.01). The mean (SD) IC₅₀ values of vitamin K₂ on the proliferation of

PBMCs of these two groups were 15.37 (30.05) and 20.67 (25.87) μ M, respectively, and the values in PBMCs of the patients were significantly lower than those in PBMCs of the healthy subjects ($P=0.0179$) (**Table 2, Figure 3b**).

3.3. Relationship between the IC_{50} values of vitamin K_2 and immunosuppressive drugs on the proliferation of PBMCs of pediatric AD patients

In order to evaluate relationships in anti-proliferative efficacies on PBMCs of pediatric AD patients between vitamin K_2 and immunosuppressive drugs, we related the IC_{50} values of vitamin K_2 and those of three typical immunosuppressive drugs by Pearson's coefficient correlation test (**Figure 4**). There was a significant correlation between the IC_{50} values of vitamin K_2 and those of tacrolimus (**Figure 4b**, $P=0.0001$, $r=0.8871$). Meanwhile there was no significant correlation between the IC_{50} values of vitamin K_2 and those of cyclosporine A or methylprednisolone (**Figures 4a and 4c**).

3.4. Relationship between the PBMC-suppressive potencies of the drugs and clinical parameters of pediatric AD patients

In order to examine the relationship between the drug effects on PBMCs and characteristics or laboratory data of the pediatric AD patients, the IC_{50} values of vitamin K_2 , cyclosporine A, tacrolimus and methylprednisolone were analyzed for their relationship with each of 7 factors of the patients, i.e., ages, proliferation stimulation indices by mitogen, scorings of atopic dermatitis (SCORAD), blood eosinophil counts, serum total IgE concentrations, specific IgE in serum (Dermatophagoides pteronissinus) concentrations and thymus and activation-regulated chemokine concentrations (TARC) (see **Table 1**). There was a significant correlation between the IC_{50} values of vitamin K_2 and the blood eosinophil counts ($P=0.0099$, $r=0.7086$). The IC_{50} values of tacrolimus and blood eosinophil counts also correlated significantly ($P=0.0032$, $r=0.7722$) (see **Table 3**). Whereas, no correlation has been observed between the IC_{50} values of these drugs and other characteristics or laboratory data of the patients.

4. Discussion

Our results showed that vitamin K₂ inhibited the proliferation of PBMCs of both pediatric AD patients and healthy subjects dose-dependently (**Figure 3a**). Considering the immunosuppressive efficacies of vitamin K₃ and K₅, as we reported previously ¹⁶, naphthoquinone skeleton is suggested to be an important chemical unit to develop new immunosuppressant with less side effects, especially for pediatric patients with immunological diseases, such as AD ⁹.

According to our present data, pediatric AD patients were likely to be more sensitive towards vitamin K₂ and the immunosuppressive drugs such as cyclosporine A and methylprednisolone (**Figure 2 and Figure 3b**). The mean (SD) ages of the AD patients we examined in this study were significantly lower than that of the healthy subjects, and thus, the difference of ages should be taken into account to evaluate the individual sensitivity of PBMCs to the immunosuppressive agents in pediatric patients ¹⁷. In this regard, however, the IC₅₀ values of the immunosuppressive drugs or vitamin K₂ were not significantly correlated with ages (**Table 3**), suggesting that the PBMC sensitivity to the immunosuppressive efficacy of these agents were not age-dependent. Comparing with the effect of tacrolimus, external use of vitamin K₂ would be an alternative strategy to manage pediatric AD, since the strong correlation between IC₅₀ values of vitamin K₂ and tacrolimus on the PBMCs of AD patients was observed ($P=0.0001$, $r=0.8871$; **Figure 4b**).

Eosinophilia is one of the typical features in many of AD patients. Because eosinophilia changes more rapidly than serum IgE concentration, blood eosinophil count has been reported to be an index to assess changes of the AD condition ⁵. Our present study also revealed that there was a strong and significant correlation between the blood eosinophil counts of the pediatric AD patients and the individual pharmacodynamics of tacrolimus and vitamin K₂ on PBMCs (**Table 3**). Comparing with cyclosporine A and steroids, these data suggested that it is of value to pay more attention to the relationship of the therapeutic dose of tacrolimus and blood eosinophil count in patients, combining with the clinical symptoms of AD. The relationship between the

immunosuppressive effect of vitamin K₂ and eosinophil count is another point which should be addressed.

T cells and T cell-derived cytokines partly contribute to the initiation of AD pathophysiology¹. The skin lesions of AD patients are infiltrated by activated T cells. Then eosinophils and antigen-presenting Langerhans cells bind and present IgE-complexed allergens on their surface. In addition, activation of peripheral blood T cells preferentially secretes Th 2 cytokines and help B cells to produce large amounts of IgE which is associated with this skin disease¹⁸. Therefore, inhibiting T cell proliferation and T cell-derived cytokine secretion would be helpful to improve the AD symptoms of patients. The results of MTT assays showed that 10 μ M of vitamin K₂ inhibited the proliferation of PBMCs of pediatric patients (**Figure 3a**). This finding is consistent with the results from Myneni *et al*¹⁹. One possible mechanism is that vitamin K₂ inhibits the activation of NF- κ B through the repression of IKK α / β phosphorylation, because NF- κ B plays critical role in the T cell development^{20, 21}. Our previous finding also showed that vitamin K₂ could change the balance of Th1 and Th2 in PBMCs of pediatric AD patients *in vitro*⁹. Specifically, the ratio of IL-4/IL2 decreased from 3.4 to 0.7, whereas the ratio of IL-4/IFN- γ increased from 0.001 to 0.0025 after treatment by 100 μ M of vitamin K₂⁹. The median (range) of vitamin K₂ IC₅₀ values against the proliferation of pediatric AD patient PBMCs was 3.08 (0.10~100.00) μ M (**Table 2**). According to the clinical investigation, 45 mg/day of vitamin K₂ taken orally could achieve maximum blood concentration of 40 to 50 μ M¹⁹. Considering the lipid-water partition coefficient of vitamin K₂, as like tacrolimus ointments, dermatologic topical remedy is another possible choice for AD patients. From the view point of controlling T cell proliferation and cytokine production, vitamin K₂ is a potential immunosuppressant for AD patients.

PBMCs include T lymphocytes, B lymphocytes and other mononuclear immune cells. Concanavalin A is a non-specific mitogen, and most of T lymphocytes will be stimulated by this mitogen. On the other hand, comparing with concanavalin A, anti-CD3 antibody appears to be more suitable for analyzing the T cell signaling pathway because the target of which is a specific T

cell antigen. In the present study, concanavalin A -stimulated PBMCs were used to evaluate the pharmacological parameters of immunosuppressive drugs *in vitro* because this PBMC culture procedures can simulate the complicated immune system *in vivo* in several autoimmune disorders. Accordingly, the results obtained *in vitro* by use of this PBMC culture system stimulated by concanavalin A were significantly correlated with the clinical efficacies of these drugs in our previous studies ²²⁻²⁵. However, we are looking forward to hearing the new reports of animal experiments which may continuously reveal us the anti-AD effects of vitamin K₂ *in vivo*.

5. Conclusions

Vitamin K₂ inhibited T cell mitogen-stimulated proliferation of PBMCs from pediatric AD patients dose-dependently. PBMCs from pediatric AD patients were more sensitive to the immunosuppressive efficacy of vitamin K₂, comparing with the PBMCs from healthy subjects. The individual immunosuppressive pharmacological efficacies of vitamin K₂ and tacrolimus could be inferred from the blood eosinophil count of the pediatric AD patients.

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Disclosure

The authors declare that they have no conflicts of interest.

Author contribution

K. S. designed and directed the study; W. X. and K. M. performed the experiments and wrote the manuscript; and T. M., S. S., M. C., and H. K. collected clinical data and provided the blood

samples of patients; and H. W. and S. T. gave technical support; and T. H. gave conceptual advice. All of the authors critically reviewed and approved the final manuscript.

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Table 1. Characteristics and laboratory data of pediatric patients with atopic dermatitis

Case	Gender	Age (years)	SCORAD	Blood eosinophil count (/μL)	Serum total IgE (U/mL)	Specific IgE in serum (Dermatophagoides pteronisus) (U _A /mL)	TARC (pg/mL)	Stimulation index †
1	M	5.4	33.5	460	1210	98.8	1933	4.22
2	M	9.4	10.5	625	3850	100	341	2.60
3	M	1.5	20	272	148	0.1	401	6.41
4	M	3.2	15	40	323	0.1	564	7.63
5	M	4.8	40	391	1020	100	598	3.93
6	F	2.1	32.5	1120	1010	100	965	2.06
7	M	2.8	10	456	374	69.3	-	5.21
8	M	12.2	10	606	1450	98.9	455	2.70
9	F	10	23	511	703	100	278	3.47
10	M	3.7	-	181	644	83.3	-	2.36
11	M	12.8	-	118	585	10.0	331	4.78
12	M	4.4	13.5	266	94	25.4	702	4.17

Mean±SD	6.0±3.8	20.8±10.5	421±276	951±963	65.5±41.4	657±468	4.13±1.62
Median	4.6	17.5	424	674	91.1	510	4.05
Minimum	1.5	10	40	94	0.1	278	2.06
Maximum	12.8	40	1120	3850	100	1933	7.63

Abbreviated symbols: SCORAD, scoring of atopic dermatitis; TARC, thymus and activation-regulated chemokine. †PBMC proliferation ratio between cells cultured in the presence and absence of concanavalin A (see Materials and Methods).

Table 2. IC₅₀ values of drugs on concanavalin A-stimulated proliferation of PBMCs of pediatric patients with atopic dermatitis and healthy subjects

Case	Pediatric patients with atopic dermatitis				Healthy subjects			
	IC ₅₀ values of cyclosporine A (ng/mL)	IC ₅₀ values of tacrolimus (ng/mL)	IC ₅₀ values of methylprednisolone (ng/mL)	IC ₅₀ values of vitamin K2 (μM)	IC ₅₀ values of cyclosporine A (ng/mL)	IC ₅₀ values of tacrolimus (ng/mL)	IC ₅₀ values of methylprednisolone (ng/mL)	IC ₅₀ values of vitamin K2 (μM)
1	3.70	0.02	0.77	50.04	1461.80	0.62	140.26	19.41
2	1.00	0.01	0.53	0.10	332.97	0.01	83.06	5.48
3	2.16	0.02	0.10	0.10	46.90	0.01	21.22	100.00
4	1.00	0.01	1.17	1.33	151.80	0.01	10.86	24.27
5	2.47	0.04	6.86	3.01	6.85	0.01	18.12	17.85
6	0.01	1.00	2.00	100.00	15.12	0.01	2.83	4.05
7	1.64	0.02	1.83	5.29	15.73	0.04	4.25	11.77
8	1.47	0.03	4.44	4.13	0.01	0.01	0.42	12.01

9	3.06	0.02	1.96	3.14	85.48	0.34	7.38	8.51
10	1.00	0.01	0.10	2.56	68.51	0.28	166.36	20.96
11	3.93	0.01	0.10	13.86	56.27	0.14	5.79	18.20
12	2.55	0.01	13.10	0.90	235.41	0.81	46.00	5.51
Mean±SD	2.00±1.19	0.10±0.28	2.75±3.83	15.37±30.05	206.40±408.19	0.19±0.27	42.21±57.20	20.67±25.87
Median	1.90	0.02	1.50	3.08	62.39	0.03	14.49	14.93
Minimum	0.01	0.01	0.10	0.10	0.01	0.01	0.42	4.05
Maximum	3.93	1.00	13.10	100.00	1461.79	0.81	166.36	100.00

Table 3. Relationship between IC₅₀ values of drugs on PBMC proliferation and subject-related factors as analyzed by Pearson's coefficient correlation test

Drugs	Statistical Paramete r	Patient-related factors							Healthy subject-related factors	
		Age	Stimulatio n index	SCORA D	Blood eosinophi l count	Serum total IgE	Specific IgE in serum (Dermatophagoid es pteronisinus)	TARC	Age	Stimulatio n index
vitamin K2	r	-0.2454	-0.3510	0.5357	0.7086	0.0302 5	0.3246	0.6048	-0.3240	0.2607
	p	0.4421	0.2632	0.1105	0.0099**	0.9256	0.3033	0.0640	0.3042	0.4132
cyclosporine A	r	0.4104	0.2388	0.2861	-0.4304	-0.2451	-0.1848	0.1779	-0.0755 2	-0.3786
	p	0.1852	0.4548	0.4229	0.1625	0.4427	0.5653	0.6230	0.8156	0.2249
tacrolimus	r	-0.3085	-0.3902	0.3885	0.7722	0.0181	0.2664	0.2217	0.2524	-0.1911

		3								
methylprednisolone	<i>p</i>	0.3293	0.2098	0.2673	0.0032**	0.9554	0.4026	0.5382	0.4287	0.5519
	<i>r</i>	-0.0608	-0.1003	-0.01623	-0.01125	-0.2149	-0.02163	-0.0101	0.3519	-0.3254
	<i>p</i>	0.8509	0.7564	0.9654	0.9723	0.5023	0.9468	0.9779	0.2620	0.3020

Abbreviated symbols: SCORAD, scoring of atopic dermatitis; TARC, thymus and activation-regulated chemokine; stimulation index represents the PBMC proliferation ratio between cells cultured in the presence and absence of concanavalin A. (see Materials and Methods). ** $p < 0.01$

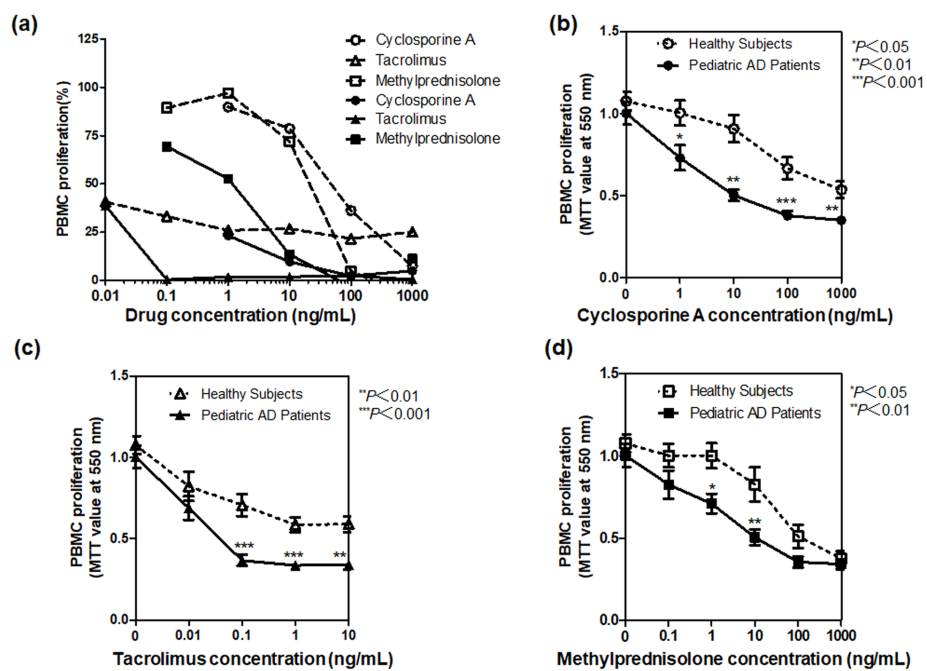
Figure legends

Figure 1. Effects of cyclosporine A, tacrolimus and methylprednisolone on the proliferation of mitogen-activated PBMCs of pediatric AD patients and healthy subjects. (a) Typical dose-response curves of cyclosporine A ($\circ$), tacrolimus (Δ) and methylprednisolone ($\square$) on proliferation of concanavalin A-stimulated PBMCs of one pediatric AD patient (full line with closed symbol) and one healthy subject (dotted line with open symbol). (b-d) Comparison of the MTT values of PBMC proliferation between the pediatric AD patients (full line with closed symbol) and healthy subjects (dotted line with open symbol). Statistical analyses were performed using Mann-Whitney test. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, as compared to the values of the same drug concentration group in PBMCs from healthy subjects.

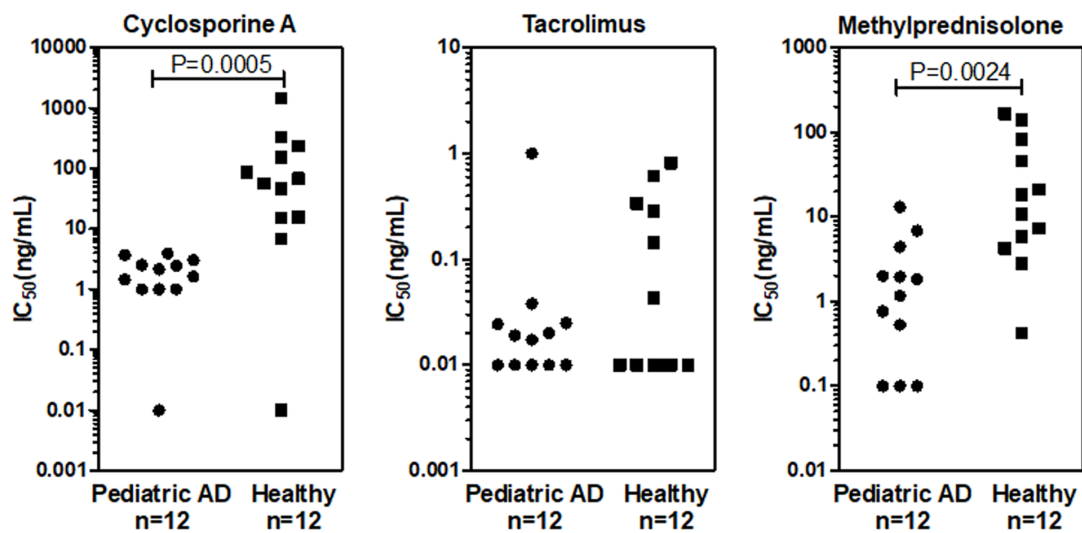
Figure 2. Comparison of the IC_{50} values of cyclosporine A, tacrolimus and methylprednisolone after the stimulation by concanavalin A between the PBMCs of the pediatric AD patients and the PBMCs of healthy subjects. The data were analyzed by Mann-Whitney test.

Figure 3. Effects of vitamin K_2 on proliferation of PBMCs of pediatric AD patients and healthy subjects. Cell proliferation was determined by MTT assay. (a) Inhibition of PBMCs by vitamin K_2 (black filled column, healthy subjects and white filled column, pediatric AD patients). The data were expressed as means $\pm$ S.E.M and analyzed by Dunnett's multiple comparison test. $^{\#}P < 0.05$, $^{###}P < 0.001$ (pediatric AD patients) and $^*P < 0.05$, $^{***}P < 0.001$ (healthy subjects), as compared to each control groups (without drug), respectively. (b) Comparison of the IC_{50} values of vitamin K_2 under the stimulation by concanavalin A between PBMCs of the pediatric AD patients and the healthy subjects. The data were analyzed by Mann-Whitney test. (n=12)

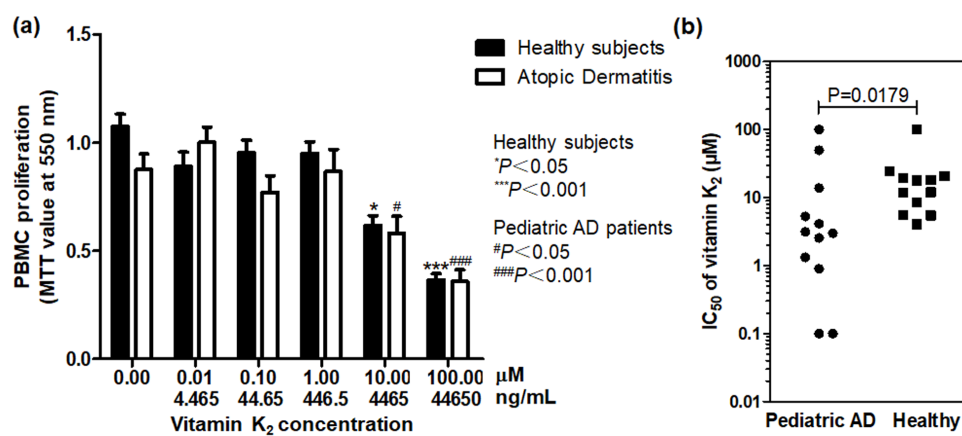
Figure 4. Relationship between the IC_{50} values for cyclosporine A (a), tacrolimus (b) or methylprednisolone (c) and the IC_{50} values of vitamin K_2 in concanavalin A-stimulated PBMCs of pediatric AD patients. The data were analyzed by Pearson's coefficient correlation test.



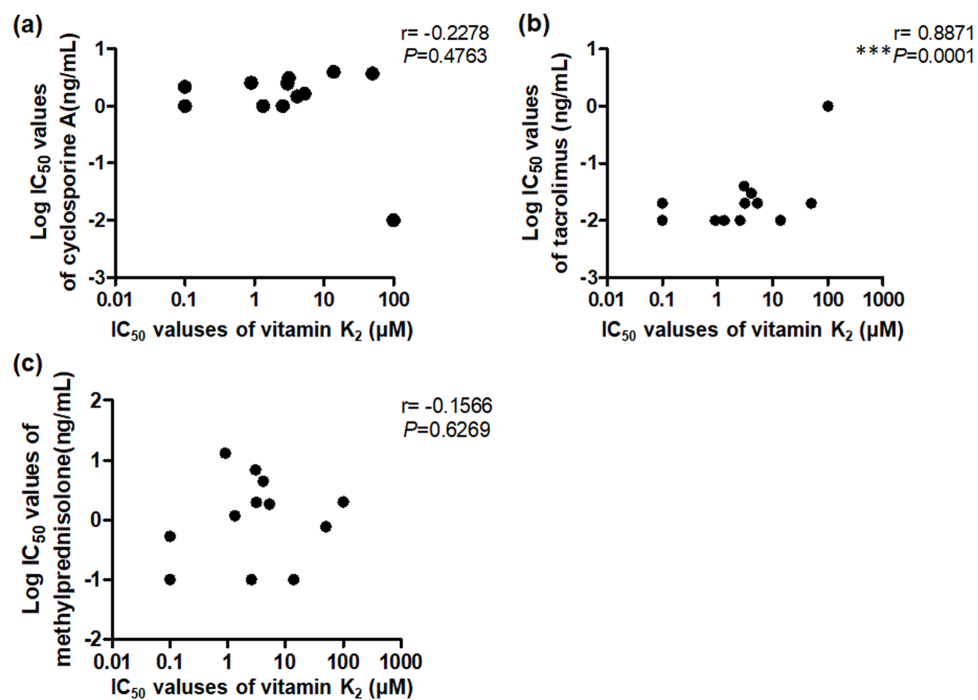
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