



# Vitamin D deficiency during pregnancy affects the function of Th1/Th2 cells and methylation of IFN- $\gamma$ gene in offspring rats

XianTing Jiao<sup>a,1</sup>, Lei Wang<sup>a,1</sup>, ZhenZhen Wei<sup>c,1</sup>, Bin Liu<sup>d</sup>, XiaoYan Liu<sup>d</sup>, XiaoDan Yu<sup>b,\*</sup>

<sup>a</sup> MOE-Shanghai Key Lab of Children's Environmental Health, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

<sup>b</sup> Department of Developmental and Behavioral Pediatrics, Pediatric Translation Medicine Institute, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China

<sup>c</sup> Department of Respiration, Children's Hospital of Shanghai, Shanghai Jiao Tong University School of Medicine, Shanghai 200040, China

<sup>d</sup> College of Food Science, Fujian Agriculture and Forestry University, Fuzhou 350002, China

## ARTICLE INFO

### Keywords:

Vitamin D deficiency  
Pregnancy  
Th1 cell  
Th2 cell  
IFN- $\gamma$  methylation

## ABSTRACT

The effects of maternal vitamin D status on offspring's Th1/Th2 cell function and the related mechanisms have not been reported. In this study, we established the rat model of vitamin D deficiency during pregnancy. 48 female Sprague-Dawley rats (8 weeks old) were randomly assigned to three groups (n = 16/group): control group (fed with standard AIN-93 G diet until parturition), vitamin D deficiency group (VDD group, fed with vitamin D deficient diet until parturition) and vitamin D supplementation group (VDS group, fed with vitamin D deficient diet prior to mating and with standard AIN-93 G diet during pregnancy). At 4 weeks of age, the ratio of T helper type 1/ T helper type 2 (Th1/Th2) cells and the levels of Th1/Th2 cytokines (IFN- $\gamma$ , IL-4, IL-5, IL-6, IL-10 and IL-13) in offspring rats were determined by Flow Cytometry and Meso Scale Discovery, respectively. Furthermore, DNA methyltransferase (DNMT) activity as well as the methylation levels of IFN- $\gamma$  and IL-4 genes were measured. As a result, rats in the VDD group showed a significant decrease in Th1/Th2 ratio and IFN- $\gamma$  level and an increase in IL-4 level. Additionally, up-regulated DNMT activity and increased methylation rate of IFN- $\gamma$  gene was shown in VDD offspring rats. Supplementation with vitamin D during pregnancy reversed the above abnormalities. In conclusion, maternal vitamin D deficiency affected the function of Th1/Th2 cells and methylation of IFN- $\gamma$  gene in offspring rats. Meanwhile, maternal vitamin D deficiency had the potential to regulate DNMT activity, which may determine the status of methylation.

## 1. Introduction

The incidence of childhood allergic diseases is dramatically rising. Concurrent with the increase in allergic diseases, the prevalence of vitamin D deficiency during pregnancy has been increasing [1,2], and reversing this trend itself seems to be unlikely [3,4]. Accumulating evidence indicates that maternal vitamin D status is associated with allergic diseases including wheezing, asthma, atopy and food allergy [5,6], but the underlying molecular mechanism remains unknown.

Data from experimental studies demonstrates that vitamin D has the capability to modulate immune responses. In rats challenged with LPS, pretreatment with 125-(OH)<sub>2</sub>D<sub>3</sub> downregulated gene expression of T helper type 1 (Th1) and up-regulates gene expression of T helper type 2 (Th2) [7]. Similarly, in vitro study showed that treatment of T cells with calcitriol or analogs could inhibit the production of Th1 cytokines [IL-2,

interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )], but promotes the secretion of Th2 cytokines (IL-3, IL-4, IL-5, IL-10) [8]. It was also reported that 125(OH)<sub>2</sub>D<sub>3</sub> inhibited IFN- $\gamma$  production as well as IL-4 and IL-13 expression in human cord blood T cells [9]. A high ratio of Th2/Th1 (high Th2- and low Th1-associated chemokine levels) at birth has been shown to be associated with allergic disease and sensitization in infancy [10]. Since pregnancy is one of the critical windows during which unfavorable factors can modify fetal immune development [11], we speculate that the vitamin D level during pregnancy may affect offspring's immune function by altering the ratio of Th1/Th2. However, the effects of maternal vitamin D status on the ratio and function of Th1/Th2 cells in offspring have not been reported.

The epigenetic mechanism provides new insights into how the uterine environment may mediate the postnatal susceptibility to immune diseases [11]. Among epigenetic mechanisms, DNA methylation

\* Corresponding author at: Department of Developmental and Behavioral Pediatrics, Pediatric Translation Medicine Institute, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China.

E-mail addresses: [xd\\_yu2003@126.com](mailto:xd_yu2003@126.com), [yuxiaodan@scmc.com.cn](mailto:yuxiaodan@scmc.com.cn) (X. Yu).

<sup>1</sup> These authors contributed equally to this work.

<https://doi.org/10.1016/j.imlet.2019.06.012>

Received 17 April 2019; Received in revised form 23 June 2019; Accepted 26 June 2019

Available online 28 June 2019

0165-2478/ © 2019 European Federation of Immunological Societies. Published by Elsevier B.V. All rights reserved.

is of great significance as it alters transcription activity through certain modifications, which can manipulate gene expression directly [12]. Recently, a genome-wide methylation study reported that vitamin D deficiency was related to methylation changes in leukocyte DNA [13]. In mammals, DNA methylation is under the regulation of DNA methyltransferase (DNMT) activity, and the process can be modulated by a variety of molecular interactions and modifications [14]. Evidence suggests that vitamin D interacts with the epigenome on multiple levels, including coactivator and corepressor proteins as well as a large number of genes involved in chromatin modifications [15]. Considering the effect of 125(OH)<sub>2</sub>D<sub>3</sub> on DNA methylation, an important issue to address is that whether maternal vitamin D affects the activity of DNMT in offspring. On the other hand, DNA methylation in early-life time is necessary for regulation of several biological processes, such as cell cycle, differentiation and genomic imprinting [14]. For example, methylation of CpG sites in the IFN- $\gamma$  (the main Th1 cytokines) and IL-4 (the main Th2 cytokines) genes is crucial to Th1/Th2 differentiation and related immune response pattern [16,17]. Thus, we wonder whether maternal vitamin D status may affect the methylation of the IFN- $\gamma$  and IL-4 genes. To our knowledge, no similar studies has been conducted to explore the methylation mechanism underlying the alteration of offspring's Th1/Th2 cell function associated with maternal vitamin D levels.

To fill the gap, we performed this study to validate the effects of maternal vitamin D status on Th1/Th2 cell function in offspring rats and tried to explore the possible epigenetic mechanisms.

## 2. Materials and methods

### 2.1. Animals and diets

Experimental animal protocols and procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 6–23, revised 1985) and were approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University of Medicine (Approval No. XHEC-F-2013-173).

48 female Sprague-Dawley rats (8 weeks old) were purchased from Sino British Sippr/BK Laboratory Animal Company LTD (Shanghai, China) (license number: SCXK-2002-008). The rats were maintained in hanging cages in a controlled temperature (22  $\pm$  0.5°C) with a 12 h dark/light cycle (light on from 08:00–20:00 h). We randomly assigned these rats to three groups (n = 16/group): control group (C group), vitamin D deficiency group (VDD group) and vitamin D supplementation group (VDS group). The C group was fed with standard AIN-93 G diet (17.8% protein, 64.3% carbohydrate, 7% fat) containing vitamin D<sub>3</sub> (800 IU/kg). The VDD and VDS groups were fed with vitamin D deficient diet (17.8% protein, 64.3% carbohydrate, 7% fat) not containing vitamin D<sub>3</sub>. Four weeks later, two female rats were housed overnight with one male rat. In the next morning, female rats were checked and the presence of a vaginal plug was designated as gestational day 1. During pregnancy, maternal rats in C group and VDS group were fed with standard AIN-93 G diet while maternal rats in the VDD group were fed with vitamin D deficient diet. At parturition, 12 maternal rats were retained in each group and litters were culled to four (two males and two females, 48 offspring rats in each group) using a random number table. After parturition, all three groups were fed with standard AIN-93 G diet.

### 2.2. Serum 25(OH)D measurement

At four weeks of age, the offspring rats were anesthetized and then blood was drawn from the heart through a syringe. Serum was separated and stored in lightproof containers at -80°C until the samples were assayed for vitamin D metabolites. We used the sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) analytical method to detect serum 25(OH)D, as reported by van den Ouweland, et al [18].

The serum samples (100  $\mu$ l) were deproteinised and precipitated using methanol, acetonitrile, zinc sulfate, and internal standards that included deuterated 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> (Sigma USA). Chromatographic separations were achieved on an Agilent Poroshell 120 EC-C18 (50  $\times$  2.1 mm, 2.7  $\mu$ m) column with a gradient of water and methanol (containing 0.1% formic acid) as the mobile phase at a flow rate of 0.5 mL/min. Multiple reaction monitoring (MRM) of the analytes was performed under electrospray ionization (ESI) in the positive mode at m/z 401.3 $\rightarrow$ 383.2 and 401.3 $\rightarrow$ 159.1 for 25(OH)D<sub>3</sub>, m/z 413.3 $\rightarrow$ 395.3 and 413.3 $\rightarrow$ 355.2 for 25(OH)D<sub>2</sub>, and m/z 404.3 $\rightarrow$ 386.3 and 416.4.3 $\rightarrow$ 398.3 for d<sub>3</sub>-25(OH)D<sub>3</sub> and d<sub>3</sub>-25(OH)D<sub>2</sub>, respectively.

### 2.3. Flow cytometry

Flow Cytometry analyses were performed according to the previous study [19]. To stimulate peripheral T lymphocytes, 500  $\mu$ l of PRMI1640 and 2  $\mu$ l of Leukocyte Activated Cocktail with BD Golgiplug (BD, USA) were added to 500  $\mu$ l of whole blood sample. Then the mixture was incubated at 37 °C with 5% CO<sub>2</sub> for 5 h. Cells were firstly stained with anti-CD3-FITC and anti-CD8a-Alexa Fluor<sup>®</sup>647. Next, the cells were permeabilized using the Fix/Perm solution (BD, USA) and stained with anti-IFN- $\gamma$ -PE and anti-IL-4-PE after fixation. All the antibodies used above were purchased from BD (USA) and incubated for 30 min at 4°C in the dark. Finally, cells were resuspended in phosphate buffer saline (PBS) and analyzed using CytoFlex S (Beckman Coulter, USA) within 1 h. Appropriate isotype controls and single staining controls were used.

### 2.4. Determination of cytokines

Quantitative analysis of interferon-gamma (IFN- $\gamma$ ), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-13 (IL-13) levels in serum was performed using the MSD<sup>®</sup> Cytokine Assays Proinflammatory Panel 2 Kits (MSD, USA). MSD plates were analyzed on the MS2400 imager (MSD, USA).

### 2.5. DNMT activity assay

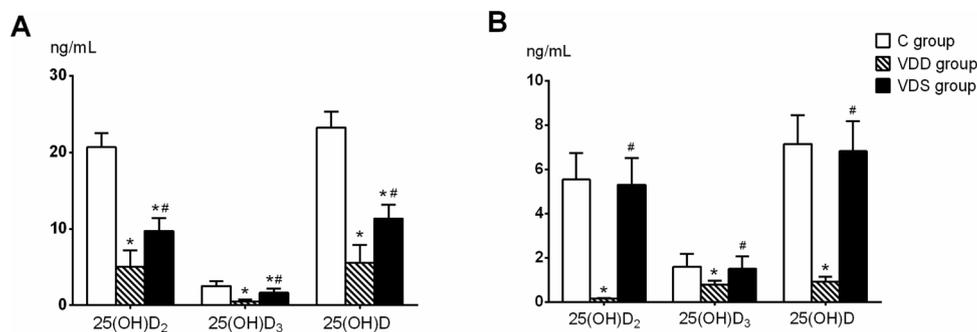
Total DNA methyltransferase was measured according to the previous study [20]. The liver nuclear extract was prepared with the EpiQuik Nuclear Extraction Kit (Epigentek, USA). The activity of DNMT was measured using the EpiQuik DNA Methyltransferase Activity/Inhibition Assay Kit (Epigentek, USA) following the manufacturer's instruction.

### 2.6. DNA methylation analysis

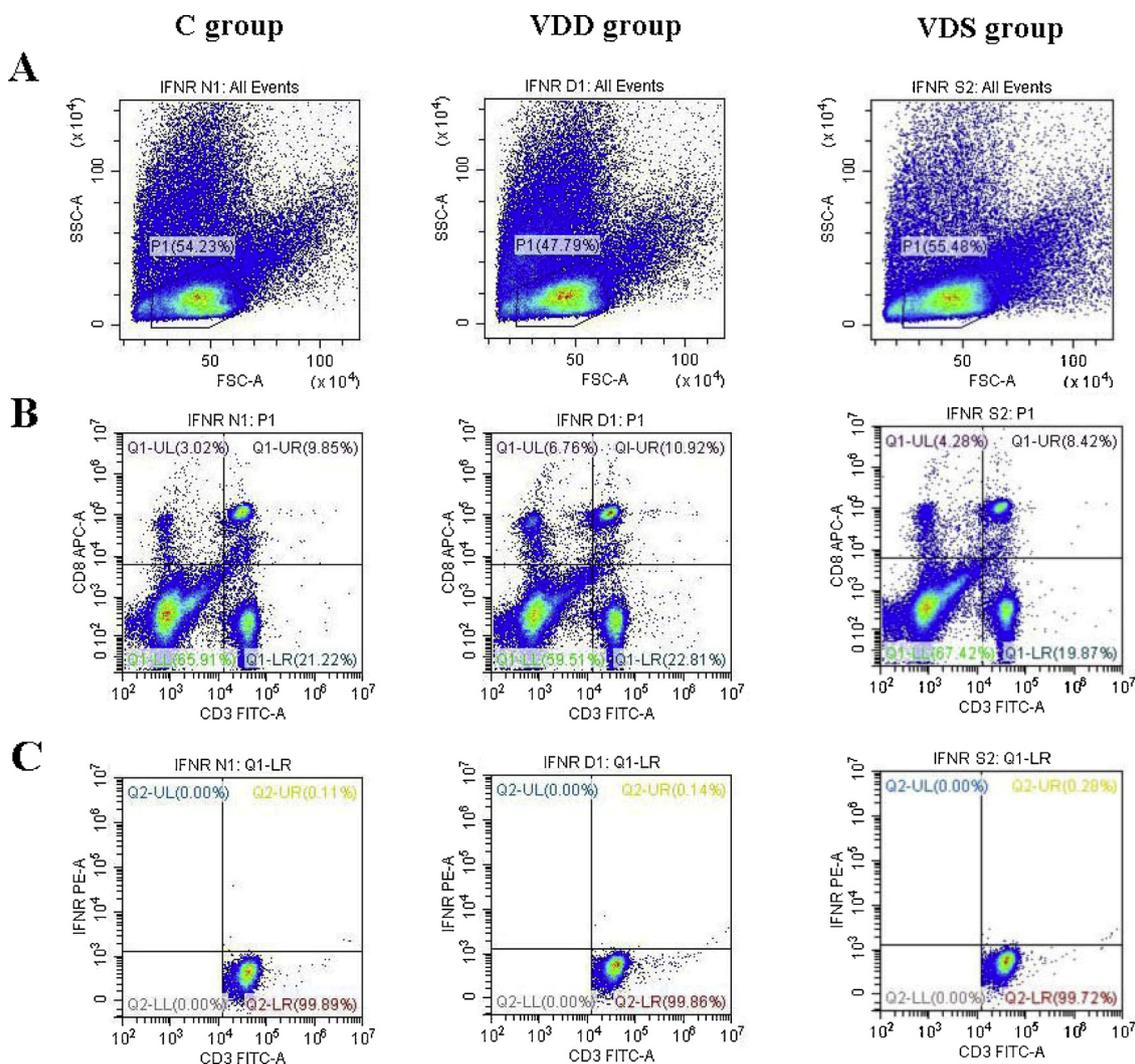
High-throughput quantitative DNA methylation analysis using the MassARRAY EpiTYPER platform (Sequenom, Inc, USA) was performed as previously described [21]. Genomic DNA was extracted from blood sample using the DNA extraction kit (BioTeKe Corporation, China) and DNA concentration was determined by spectrophotometry. The IFN- $\gamma$  and IL-4 primer pairs were designed using the EpiDesigner tool from Sequenom and used in the polymerase chain reaction (PCR) of bisulfite-treated gDNA. The primer sequences are shown in Table 1. PCR conditions were 94 °C for 4 min, followed by 45 cycles of 94 °C for 20 s,

**Table 1**  
PCR primers for IFN- $\gamma$  and IL-4.

Region		Sequence (5'-3')
IFN- $\gamma$	forward	aggaagagagTATATTGTAAGGGTTAAAAGGGGGA
	reverse	cagtaatcagcactactataggagaaggct CCATAAAACAAAACACTACAAAACAAA
IL-4	forward	aggaagagagTGTAAAGGTTGGGTAGGATTTAGA
	reverse	cagtaatcagcactactataggagaaggct CATCTCTCAAACACCCAAATAATA



**Fig. 1.** Serum levels of vitamin D metabolites in maternal (A, n = 12 per group) and offspring (B, n = 48 per group) rats. \*denotes a significant difference compared to the C group (P < 0.05). #denotes a significant difference compared to the VDD group (P < 0.05).



**Fig. 2.** Flow cytometric evaluation of Th1 cells in PBMCs of three groups. (A) Lymphocytes. FSC/SSC were gated. (B) CD3<sup>+</sup> CD8<sup>-</sup> T lymphocytes. CD3/CD8 were gated. (C) Intracellular expression of IFN-γ on CD3<sup>+</sup> CD8<sup>-</sup> T cells.

56 °C for 30 s, and 72 °C for 1 min, with a final extension of 3 min at 72 °C. Each amplicon was visualized on a 1.5% agarose gel. Subsequently, 1.7 μL of RNase-free water and 0.3 μL of shrimp alkaline phosphatase (SAP) Enzyme were added to the PCR products and incubated at 37 °C for 20 min and then at 85 °C for 5 min to dephosphorylate all unincorporated deoxynucleotide triphosphates (dNTPs).

In vivo transcription and the T-cleavage reaction were performed at the same time using all reagents from Sequenom for 3 h at 37 °C. After adding Clean Resin (Sequenom, Inc, USA), the cleavage reaction products were spotted robotically onto a 384-element SpectroCHIP<sup>®</sup> bioarray (Sequenom, Inc, USA) using the MassARRAY Nanodispenser (Sequen *immunology letters* om, Inc, USA). Matrix-assisted laser

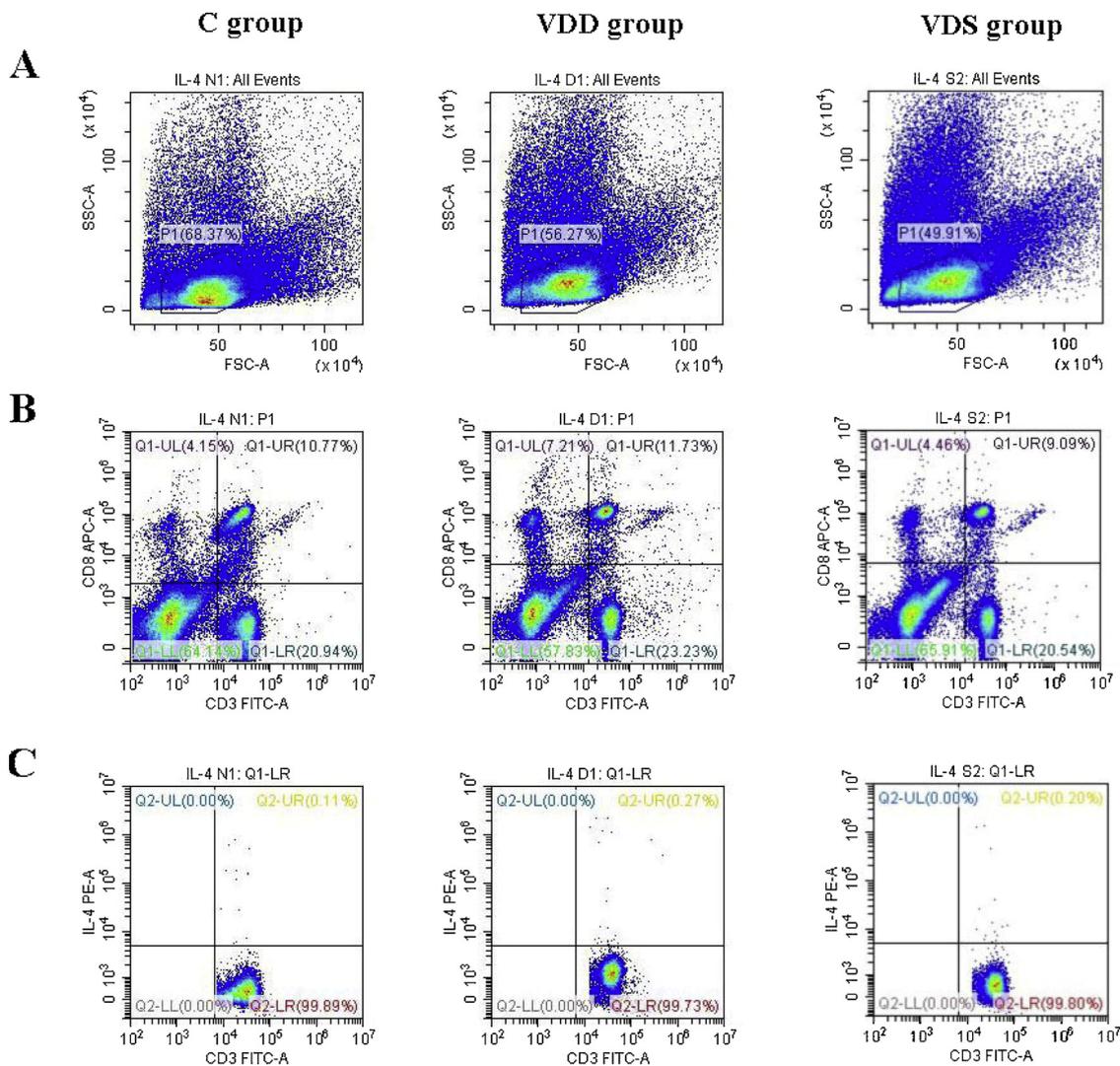


Fig. 3. Flow cytometric evaluation of Th2 cells in PBMCs of three groups.

- (A) Lymphocytes. FSC/SSC were gated.
- (B) CD3<sup>+</sup> CD8<sup>-</sup> T lymphocytes. CD3/CD8 were gated.
- (C) Intracellular expression of IL-4 on CD3<sup>+</sup> CD8<sup>-</sup> T cells.

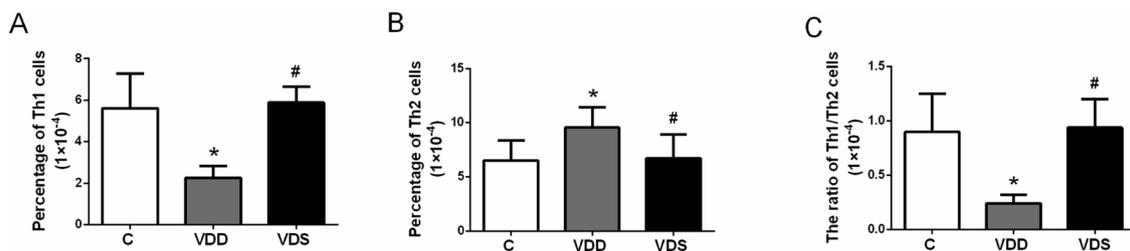


Fig. 4. The ratio of Th1/Th2 cells in peripheral blood of offspring rats (n = 6 per group).

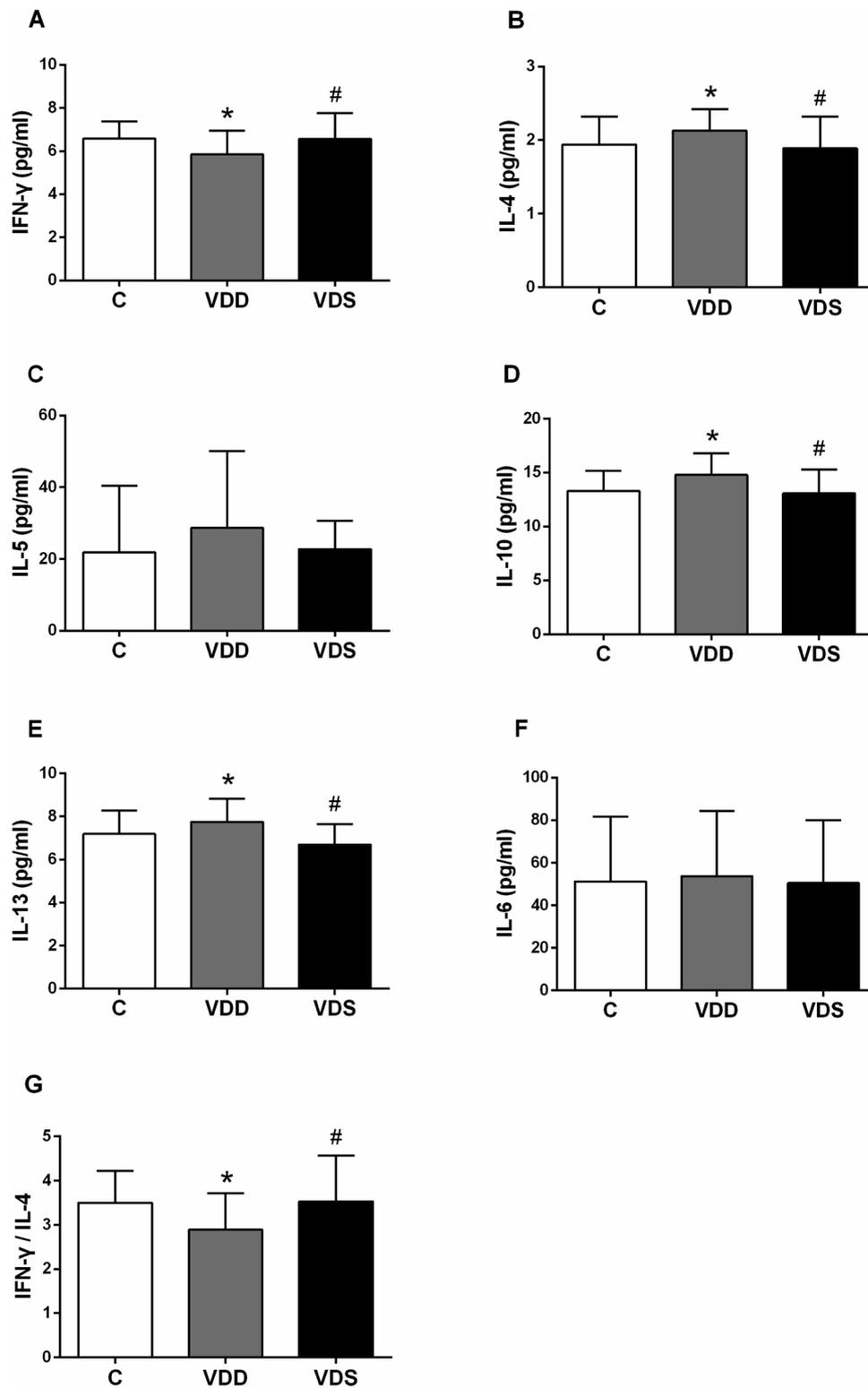
- (A) Percentage of Th1 cells.
- (B) Percentage of Th2 cells.
- (C) The ratio of Th1/Th2 cells.

\*denotes a significant difference compared to the C group (P < 0.05).  
 #denotes a significant difference compared to the VDD group (P < 0.05).

desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Sequenom, Inc, USA) was utilized to obtain the mass spectra, followed by the generation of DNA methylation data using the MassARRAY EpiTYPER™ software (Sequenom, Inc, USA).

2.7. Statistical analysis

Data were analyzed by using SPSS 20.0. Statistical analysis of flow cytometry was performed with the Student's t test. The cytokine levels are normally distributed by Kolmogorov-Smirnov normality test. The difference between groups was analyzed by t test and non-parametric



**Fig. 5.** Levels of IFN- $\gamma$  (A), IL-4 (B), IL-5 (C), IL-6 (D), IL-10 (E) and IL-13 (F) in peripheral blood (n = 6 per group). \*denotes a significant difference compared to the C group (P < 0.05). #denotes a significant difference compared to the VDD group (P < 0.05).

test. A P-value of 0.05 was considered statistically significant.

### 3. Results

#### 3.1. The model of vitamin D deficiency and supplementation during pregnancy

As shown in Fig.1A, for maternal rats, the levels of serum 25(OH)D<sub>2</sub>,

**Table 2**  
DNMT activity in liver nuclear protein extracts in offspring rats.

Group	n	DNMT activity (ng/ $\mu$ l)
C	6	3.05 $\pm$ 3.19
VDD	6	9.51 $\pm$ 8.01*
VDS	6	3.29 $\pm$ 3.24#

\* denotes a significant difference compared to the C group ( $P < 0.05$ ).

# denotes a significant difference compared to the VDD group ( $P < 0.05$ ).

25(OH)D<sub>3</sub> and 25(OH)D in C group were (20.71  $\pm$  1.82), (2.53  $\pm$  0.62) and (23.23  $\pm$  2.08) ng/ml, respectively. Compared to the C group, the levels of vitamin D metabolites in VDD group [(5.04  $\pm$  2.15), (0.49  $\pm$  0.26) and (5.52  $\pm$  2.36) ng/ml, respectively] significantly decreased ( $P < 0.05$ ). Vitamin D levels in VDS group [(9.72  $\pm$  1.65), (1.63  $\pm$  0.53) and (11.35  $\pm$  1.84) ng/ml, respectively] were significantly higher than those in VDD group but still lower than those in C group ( $P < 0.05$ ).

As shown in Fig. 1B, for offspring rats, the levels of serum 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and 25(OH)D in C group were (5.54  $\pm$  1.20), (1.60  $\pm$  0.57) and (7.15  $\pm$  1.30) ng/ml, respectively. Compared to the C group, the levels of vitamin D metabolites in VDD group [(0.15  $\pm$  0.03), (0.78  $\pm$  0.19) and (0.93  $\pm$  0.22) ng/ml, respectively] significantly decreased ( $P < 0.05$ ). Vitamin D levels in VDS group [(5.31  $\pm$  1.20), (1.50  $\pm$  0.57) and (6.82  $\pm$  1.37) ng/ml, respectively] was significantly higher than those in VDD group ( $P < 0.05$ ) but comparable with those in C group.

### 3.2. The ratio of Th1 and Th2 cells in peripheral blood

To determine the percentage of Th1 and Th2 cells, flow cytometry was conducted. Figs. 2 and 3 showed the representative flow cytometry results for Th1 cells and Th2 cells in three groups, respectively. As indicated in Fig. 4, the proportion of Th1 cells in VDD group was significantly lower than that in C group and VDS group ( $P < 0.05$ ). In contrast, the proportion of Th2 cells in VDD group was significantly higher than in C group and VDS group ( $P < 0.05$ ). As a result, the ratio of Th1/Th2 cells in VDD group was significantly lower compared with C group and VDS group ( $P < 0.05$ ). These results suggest that vitamin D deficiency in utero promotes Th2 polarization in offspring rats, and the situation can be reversed when vitamin D is supplementation during pregnancy.

### 3.3. Levels of Th1 and Th2 cytokines in peripheral blood

As demonstrated in Fig. 5A–F, the levels of main Th1 cytokines (IFN- $\gamma$ ) and main Th2 cytokines (IL-4, IL-5, IL-6, IL-10 and IL-13) were determined. Vitamin D deficiency resulted in a significant decrease in IFN- $\gamma$  (Th1 cytokines) secretion and a significant increase in IL-4, IL-10 and IL-13 (Th2 cytokines) production ( $P < 0.05$ ).

Fig. 5G shows the ratio of representative Th1-related cytokine/representative Th2-related cytokine (IFN- $\gamma$ /IL-4) in C group, VDD group and VDS group. Compared with C group, the ratio of IFN- $\gamma$ /IL-4 in VDD group significantly decreased ( $P < 0.05$ ) but was comparable in VDS group.

**Table 3**  
Methylation percentage of IFN- $\gamma$  and IL-4 in offspring rats.

Group	n	Methylation rate of IFN- $\gamma$ (> 80%)	Methylation rate of IFN- $\gamma$ (< 30%)	Methylation rate of IL-4 (> 80%)	Methylation rate of IFN- $\gamma$ (< 30%)
C	6	20.0%	26.6%	56.7%	26.7%
VDD	6	43.3%*	13.3%	46.7%	13.3%
VDS	6	20.0%#	10.0%	53.3%	3.3%

\* denotes a significant difference compared to the C group ( $P < 0.05$ ).

# denotes a significant difference compared to the VDD group ( $P < 0.05$ ).

### 3.4. DNMT activity in liver nuclear protein extracts

We investigated the DNMT activity in liver nuclear protein extract using an ELISA-like in vitro methylation assay (Table 2). The levels of liver DNMT activity in C group, VDD group and VDS group were (3.05  $\pm$  3.19), (9.51  $\pm$  8.01) and (3.29  $\pm$  3.24) ng/ $\mu$ l, respectively. Compared with C group, DNMT activity in VDD group significantly increased ( $P < 0.05$ ) and was comparable in VDS group. Thus, vitamin D supplementation during pregnancy reversed DNMT activity in offspring rats.

### 3.5. The methylation status of IFN- $\gamma$ and IL-4 genes

We evaluated the methylation of IFN- $\gamma$  (key Th1 cytokine) and IL-4 (key Th1 cytokine) genes in peripheral blood (Table 3). For IFN- $\gamma$ , the methylation rate in VDD group was significantly higher than that in C group ( $P < 0.05$ ). In VDS group, the methylation rate was significantly lower compared with VDD group. For IL-4, the difference in methylation rate between each group was not statistically significant ( $P > 0.05$ ).

## 4. Discussion

This in vivo study demonstrated that vitamin D deficiency during pregnancy caused immune alterations in offspring, as reflected by decreased ratio of Th1/Th2 cells and abnormal immune responses. Our study is the first to clarify the influence of maternal vitamin D on methylation of IFN- $\gamma$  gene by affecting DNMT activity, which may potentially lead to adverse immune outcome.

In our study, the serum 25(OH)D concentrations of VDD dams at gestational day 10 significantly decreased, suggesting that the rat model of prenatal vitamin D deficiency was successfully established. Moreover, by feeding mother rats with normal diet during pregnancy, the VDS offspring rats showed no significant difference in the 25(OH)D levels with the C group at 4 weeks of age. The results revealed that the vitamin D levels in offspring rats were highly dependent of maternal vitamin D status, which has also been illustrated in humans [22]. Our study also proved that supplementation with vitamin D during pregnancy reversed the above abnormal immune responses. This results was in line with that observed in related studies [23].

Although extensive studies have explored the effects of vitamin D on Th1/Th2 cytokines production, most of them were conducted based on in vitro models [24]. This study extends the previous findings by demonstrating that maternal vitamin D deficiency can decrease the ratio of Th1/Th2 and production of IFN- $\gamma$  and increase secretion of IL-4 in offspring rats. IL-4 is not only the major product of Th2 cells but also one of the key inducing cytokines during Th2 differentiation [25]. Similarly, IFN- $\gamma$  is a signature cytokine that plays an important role in the induction of Th1 cells [26]. In our study, the development of Th1/Th2 cells and the production of Th1/Th2 cytokines were consistent. A large body of investigations have linked the decreased ratio of Th1/Th2 cells and decreased production of IFN- $\gamma$  at birth to sensitization and eczema development during childhood [27,28]. Our results that maternal vitamin D deficiency altered offspring's Th1/Th2 ratio and cell function may provide a clue for the mechanism by which in utero vitamin D

status may affect childhood allergy. Interestingly, inadequate vitamin D nutrition has been identified as a risk factor for developing Multiple Sclerosis (MS), type one diabetes [29] and other autoimmune diseases. More recently, in a Swedish study, the 25-OH-D serum level above 30 ng/mL of pregnant women had a 61% lower MS risk than those with less than 30 ng/mL [30]. Vitamin D status during pregnancy also played a significant role in the MS risk of offspring in two other studies, in the USA [31] and in Finland [32].

In addition, DNMT activity significantly increased in VDD offspring rats and reversed to normal level by vitamin D supplementation during pregnancy, implying that the activity of DNMT in offspring rats was associated with maternal vitamin D status. Until now, the mechanisms underlying the effect of 125-(OH)<sub>2</sub>D<sub>3</sub> on DNA methylation remain unclear. Thus, our finding provided novel evidence about the mediation role of DNA methylation in the vitamin D related health effects.

Consistent with the changes in DNMT activity, the methylation level of CpG sites in the IFN- $\gamma$  gene also significantly increased in VDD offspring rats and returned to normal in VDS offspring rats. During pregnancy, methylation at IFN- $\gamma$  regulatory regions in Th1 cell induces a shift towards a pro-allergic Th2 profile to prevent fetal loss [33,34]. After birth, however, there is an increasing level of methylation in the IL-4 gene and progressive demethylation in the IFN- $\gamma$  promoter [35]. White, et al. [36] reported that DNA methylation of IL-4 and IFN- $\gamma$  promoter in cord blood CD4<sup>+</sup> T cells was different from that in adult mice, suggesting age-related changes in dominance of Th1 and Th2 subsets associated with DNA methylation. In the present study, the hypermethylation status of IFN- $\gamma$  gene persisted in VDD offspring rats, indicating that maternal vitamin D deficiency had lasting effects on the methylation of IFN- $\gamma$  gene in offspring. The differentiation of Th1/Th2 cells is regulated by networks of cytokines present in the environment and Th1/Th2 transcription factors [37,38]. Therefore, the methylation of Th1/Th2 cytokine genes (IFN- $\gamma$  and IL-4) may be associated with the differentiation of Th1/Th2 cells and then influences Th1/Th2 cytokines production. Furthermore, methylation of Th1/Th2 cytokine genes was also linked to the expression of Th1/Th2 cytokines, which in turn affects the differentiation of Th1/Th2 cells. We did not find significant difference in methylation of IL-4 gene. The possible reason is that differentiation of Th2 cells is coordinated by many factors, apart from IL-4 gene [39–41]. The exact methylation mechanism that how vitamin D affects the differentiation of Th1/Th2 cells needs further elucidation.

Our study has some limitations. First, some of the CpG sites in IFN- $\gamma$  and IL-4 promoters were not detected, thus some deviations cannot be excluded. Second, we only determined the methylation of two key genes (IFN- $\gamma$  and IL-4) related with the development of Th1/Th2 cells, which may not comprehensively reflect the effects of vitamin D on the differentiation of Th1/Th2 cells.

## 5. Conclusions

Vitamin D status during pregnancy affected the function of Th1/Th2 cells and methylation of IFN- $\gamma$  gene in offspring rats. The decreased ratio of Th1/Th2 cells and Th2 polarization may be associated with increased methylation rate of IFN- $\gamma$  gene. Moreover, maternal vitamin D had the potential to influence the status of methylation by regulating DNMT activity. And, supplementation with vitamin D during pregnancy reversed the above abnormalities.

## Authors' contributions

XJ contributed significantly to analysis and wrote the manuscript; LW and ZW conceived and performed the experiments; BL and XL helped perform the analysis with constructive discussions; XY designed the experiments and approved the final version.

## Declaration of conflict of interest

The author declare no conflict of interest.

## Funding

This work was supported by the Chinese National Natural Science Foundation (grant nos. 81773411 and 81373004), National Key Research Program of China (grant no. 2016YFC1305204), Shanghai Municipal Education Commission—Gaofeng Clinical Medicine Grant Support (grant no. 20152220) and Shanghai Children's Health Services Capacity Program (grant no. GDEK201708).

## References

- [1] Y.H. Chen, L. Fu, J.H. Hao, Z. Yu, P. Zhu, H. Wang, Y.Y. Xu, C. Zhang, F.B. Tao, D.X. Xu, Maternal vitamin D deficiency during pregnancy elevates the risks of small for gestational age and low birth weight infants in Chinese population, *J. Clin. Endocrinol. Metab.* 100 (5) (2015) 1912–1919.
- [2] H. Wang, Y. Xiao, L. Zhang, Q. Gao, Maternal early pregnancy vitamin D status in relation to low birth weight and small-for-gestational-age offspring, *J. Steroid Biochem. Mol. Biol.* 175 (2018) 146–150.
- [3] G.W.K. Wong, J. Li, Y.X. Bao, J.Y. Wang, T.F. Leung, L.L. Li, J. Shao, X.Y. Huang, E.M. Liu, K.L. Shen, Y.Z. Chen, Pediatric allergy and immunology in China, *Pediatr. Allergy Immunol.* 29 (2) (2018) 127–132.
- [4] R.L. Peters, J.J. Koplin, L.C. Gurrin, S.C. Dharmage, M. Wake, A.L. Ponsonby, M.L.K. Tang, A.J. Lowe, M. Matheson, T. Dwyer, K.J. Allen, The prevalence of food allergy and other allergic diseases in early childhood in a population-based study: healthnuts age 4-year follow-up, *J. Allergy Clin. Immunol.* 140 (1) (2017) 145–153 e8.
- [5] C.A. Camargo Jr., T. Ingham, K. Wickens, R. Thadhani, K.M. Silvers, M.J. Epton, G.I. Town, P.K. Pattemore, J.A. Espinola, J. Crane, Cord-blood 25-hydroxyvitamin D levels and risk of respiratory infection, wheezing, and asthma, *Pediatrics* 127 (1) (2011) e180–7.
- [6] H. Feng, P. Xun, K. Pike, A.K. Wills, B.L. Chawes, H. Bisgaard, W. Cai, Y. Wan, K. He, In utero exposure to 25-hydroxyvitamin D and risk of childhood asthma, wheeze, and respiratory tract infections: a meta-analysis of birth cohort studies, *J. Allergy Clin. Immunol.* 139 (5) (2017) 1508–1517.
- [7] X.P. Qi, P. Li, G. Li, Z. Sun, J.S. Li, 1,25-dihydroxyvitamin D(3) regulates LPS-induced cytokine production and reduces mortality in rats, *World J. Gastroenterol.* 14 (24) (2008) 3897–3902.
- [8] S. Ardizzone, A. Cassinotti, D. Trabattoni, G. Manzionna, V. Rainone, M. Bevilacqua, A. Massari, G. Manes, G. Maconi, M. Clerici, G. Bianchi Porro, Immunomodulatory effects of 1,25-dihydroxyvitamin D3 on TH1/TH2 cytokines in inflammatory bowel disease: an in vitro study, *Int. J. Immunopathol. Pharmacol.* 22 (1) (2009) 63–71.
- [9] J. Pichler, M. Gerstmayr, Z. Szepefalusi, R. Urbanek, M. Peterlik, M. Willheim, 1 alpha,25(OH)2D3 inhibits not only Th1 but also Th2 differentiation in human cord blood T cells, *Pediatr. Res.* 52 (1) (2002) 12–18.
- [10] T.R. Abrahamsson, M. Sandberg Abenius, A. Forsberg, B. Bjorksten, M.C. Jenmalm, A Th1/Th2-associated chemokine imbalance during infancy in children developing eczema, wheeze and sensitization, *Clin. Exp. Allergy* 41 (12) (2011) 1729–1739.
- [11] V.S. Knopik, M.A. Maccani, S. Franco, J.E. McGeary, The epigenetics of maternal cigarette smoking during pregnancy and effects on child development, *Dev. Psychopathol.* 24 (4) (2012) 1377–1390.
- [12] Z. Wang, Q. Lu, Z. Wang, Epigenetic alterations in cellular immunity: new insights into autoimmune diseases, *Cell. Phys. Biochem. Int. J. Exp. Cell. Phys., Biochem., Pharmacol.* 41 (2) (2017) 645–660.
- [13] H. Zhu, X. Wang, H. Shi, S. Su, G.A. Harshfield, B. Gutin, H. Snieder, Y. Dong, A genome-wide methylation study of severe vitamin D deficiency in African American adolescents, *J. Pediatr.* 162 (5) (2013) 1004–1009 e1.
- [14] F. Lyko, The DNA methyltransferase family: a versatile toolkit for epigenetic regulation, *Nat. Rev. Genet.* 19 (2) (2018) 81–92.
- [15] I.S. Fetahu, J. Hobaus, E. Kallay, Vitamin d and the epigenome, *Front. Physiol.* 5 (2014) 164.
- [16] S. Yano, P. Ghosh, H. Kusaba, M. Buchholz, D.L. Longo, Effect of promoter methylation on the regulation of IFN-gamma gene during in vitro differentiation of human peripheral blood T cells into a Th2 population, *J. Immunol.* 171 (5) (2003) 2510–2516.
- [17] J. Liu, M. Ballaney, U. Al-alem, C. Quan, X. Jin, F. Perera, L.C. Chen, R.L. Miller, Combined inhaled diesel exhaust particles and allergen exposure alter methylation of T helper genes and IgE production in vivo, *Toxicol. Sci. Off. J. Soc. Toxicol.* 102 (1) (2008) 76–81.
- [18] J.M. van den Ouweland, M. Vogeser, S. Bacher, Vitamin D and metabolites measurement by tandem mass spectrometry, *Rev. Endocr. Metab. Disord.* 14 (2) (2013) 159–184.
- [19] S. Ni, S. Li, N. Yang, X. Tang, S. Zhang, D. Hu, M. Lu, Deregulation of regulatory t cells in acute-on-chronic liver failure: a rat model, *Mediators Inflamm.* 2017 (2017) 1390458.
- [20] S. Majid, A.A. Dar, V. Shahryari, H. Hirata, A. Ahmad, S. Saini, Y. Tanaka, A.V. Dahiya, R. Dahiya, Genistein reverses hypermethylation and induces active

- histone modifications in tumor suppressor gene B-Cell translocation gene 3 in prostate cancer, *Cancer* 116 (1) (2010) 66–76.
- [21] N. Truong, S.M. Chun, T.I. Kim, Y.A. Suh, S.J. Jang, Hypermethylation of adjacent CpG sites is negatively correlated with the expression of lineage oncogene ASCL1 in pulmonary neuroendocrine tumors, *Tumour Biol.* 39 (6) (2017) 1010428317706225.
- [22] X. Yu, W. Wang, Z. Wei, F. Ouyang, L. Huang, X. Wang, Y. Zhao, H. Zhang, J. Zhang, Vitamin D status and related factors in newborns in Shanghai, China, *Nutrients* 6 (12) (2014) 5600–5610.
- [23] J. Ji, H. Zhai, H. Zhou, S. Song, G. Mor, A. Liao, The role and mechanism of vitamin D-mediated regulation of Treg/Th17 balance in recurrent pregnancy loss, *Am. J. Reprod. Immunol.* 81 (6) (2019) e13112.
- [24] M. Das, N. Tomar, V. Sreenivas, N. Gupta, R. Goswami, Effect of vitamin D supplementation on cathelicidin, IFN-gamma, IL-4 and Th1/Th2 transcription factors in young healthy females, *Eur. J. Clin. Nutr.* 68 (3) (2014) 338–343.
- [25] P.C. Janson, M.E. Winerdal, O. Winqvist, At the crossroads of T helper lineage commitment-Epigenetics points the way, *Biochim. Biophys. Acta* 1790 (9) (2009) 906–919.
- [26] J. Zhu, H. Yamane, W.E. Paul, Differentiation of effector CD4 T cell populations (\*), *Annu. Rev. Immunol.* 28 (2010) 445–489.
- [27] Y. Fu, H. Lou, C. Wang, W. Lou, Y. Wang, T. Zheng, L. Zhang, T cell subsets in cord blood are influenced by maternal allergy and associated with atopic dermatitis, *Pediatr. Allergy Immunol.* 24 (2) (2013) 178–186.
- [28] S. Romagnani, The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? *Immunology* 112 (3) (2004) 352–363.
- [29] E. Hypponen, E. Laara, A. Reunanen, M.R. Jarvelin, S.M. Virtanen, Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study, *Lancet* 358 (9292) (2001) 1500–1503.
- [30] J. Salzer, G. Hallmans, M. Nystrom, H. Stenlund, G. Wadell, P. Sundstrom, Vitamin D as a protective factor in multiple sclerosis, *Neurology* 79 (21) (2012) 2140–2145.
- [31] F. Mirzaei, K.B. Michels, K. Munger, E. O'Reilly, T. Chitnis, M.R. Forman, E. Giovannucci, B. Rosner, A. Ascherio, Gestational vitamin D and the risk of multiple sclerosis in offspring, *Ann. Neurol.* 70 (1) (2011) 30–40.
- [32] K.L. Munger, J. Aivo, K. Hongell, M. Soilu-Hanninen, H.M. Surcel, A. Ascherio, Vitamin d status during pregnancy and risk of multiple sclerosis in offspring of women in the finnish maternity cohort, *JAMA Neurol.* 73 (5) (2016) 515–519.
- [33] G.P. White, E.M. Hollams, S.T. Yerkovich, A. Bosco, B.J. Holt, M.R. Bassami, M. Kusel, P.D. Sly, P.G. Holt, CpG methylation patterns in the IFN-gamma promoter in naive T cells: variations during Th1 and Th2 differentiation and between atopics and non-atopics, *Pediatr. Allergy Immunol.* 17 (8) (2006) 557–564.
- [34] T.H. Tan, J.A. Ellis, R. Saffery, K.J. Allen, The role of genetics and environment in the rise of childhood food allergy, *Clin. Exp. Allergy* 42 (1) (2012) 20–29.
- [35] B. Jones, J. Chen, Inhibition of IFN-gamma transcription by site-specific methylation during T helper cell development, *EMBO J.* 25 (11) (2006) 2443–2452.
- [36] G.P. White, P.M. Watt, B.J. Holt, P.G. Holt, Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells, *J. Immunol.* 168 (6) (2002) 2820–2827.
- [37] J. Zhu, W.E. Paul, Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors, *Immunol. Rev.* 238 (1) (2010) 247–262.
- [38] Y. Zhang, Y. Zhang, W. Gu, B. Sun, TH1/TH2 cell differentiation and molecular signals, *Adv. Exp. Med. Biol.* 841 (2014) 15–44.
- [39] K.M. Ansel, I. Djuretic, B. Tanasa, A. Rao, Regulation of Th2 differentiation and IL4 locus accessibility, *Annu. Rev. Immunol.* 24 (2006) 607–656.
- [40] T. Kaneko, H. Hosokawa, M. Yamashita, C.R. Wang, A. Hasegawa, M.Y. Kimura, M. Kitajima, F. Kimura, M. Miyazaki, T. Nakayama, Chromatin remodeling at the Th2 cytokine gene loci in human type 2 helper T cells, *Mol. Immunol.* 44 (9) (2007) 2249–2256.
- [41] D.U. Lee, S. Agarwal, A. Rao, Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene, *Immunity* 16 (5) (2002) 649–660.