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Prenatal Exposure of Rats to Staphylococcal Enterotoxin B Alters the Expression of Th1 and Th2 Cytokines via Decreased Methylation in the Spleen of Adult but not Neonatal Offspring

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ABSTRACT

Background: The methylation of IFN-γ and IL-4 genes is regarded as an epigenetic regulation that maintains the Th1 or Th2 phenotype.

Objective: To explore the influence of prenatal administration of the staphylococcal enterotoxin B (SEB) in pregnant rats, on the IFN-γ or IL-4 expression in the offspring spleen.

Methods: The SEB or PBS was administered intravenously to pregnant rats on the embryo-day 16. After normal delivery, the spleens from the fifth-day neonates and adult offspring were isolated under anesthesia. Quantitative PCR, western blot, ELISA and MeDIP-qPCR were applied to determine the levels of the splenic IFN-γ or IL-4 mRNAs, their protein levels, and methylation status, respectively.

Results: Prenatal administration of the SEB in pregnant rats decreased the levels of the splenic IFN-γ and IL-4 proteins in neonates, but increased their mRNA levels. However, prenatal administration of the SEB significantly augmented both mRNA and protein levels of the IFN-γ and IL-4 in the adult spleen. In addition, the prenatal SEB administration decreased the methylation of the splenic IFN-γ and IL-4 in adult but not neonatal offspring.

Conclusion: The prenatal administration of SEB in pregnant rats can cause a mixed Th1 and Th2 cytokines response in the offspring spleen, and alter the cytokine expression of the Th1 and Th2 via decreasing the methylation in adult but, not neonatal offspring spleen.

Keywords: Staphylococcal enterotoxin B, Gestation, Cytokine, Methylation, Offspring

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INTRODUCTION

Staphylococcal enterotoxin B (SEB) is one of the numerous exotoxins secreted by *Staphylococcus aureus* (*S. aureus*). The SEB can cause food poisoning (1), lethal toxic-shock syndrome (2), and is a potential bioterrorism threat agent (3). The SEB also functions as a superantigen. In contrast to the conventional antigens, the SEB can be directly combined with the peptide-binding groove of MHC II molecules without processing and presentation by the antigen-presenting cells, thereby stimulating the T cells which mount specific Vβ chains on the T-cell receptors (4). This recognition mode enables the SEB to stimulate a great number of the T cells, without considering anything about their antigen specificity, which provokes the production of robust cytokines (5,6). A variety of studies (7-10) have shown that the SEB can lead to high levels of many inflammatory cytokines to mediate the pathogenesis of some diseases caused by the *S. aureus*. $CD4^+$ T cells after antigen recognition can proliferate and can be differentiated into the effector cells of either type 1 T helper (Th1) or the Th2 (11, 12). The Th1 cells can produce Th1 cytokines such as the IL-2 and the IFN-γ, while the Th2 type cells can secrete Th2 cytokines like the IL-4, IL-6, and the IL-13 (13). The gene methylation of the IFN-γ and the IL-4 exerts a vital function as the regulating epigenetic characteristics and can be involved in maintaining the Th1 or the Th2 cells (14, 15).

S. aureus has been documented as a promoting issue among pregnant women (16, 17) due to its ability to reside in some sites, for instance, the genital tract and breast (18, 19). Such colonization helps *S. aureus* pass through the pregnant women to their fetus or infant. We have previously revealed that the prenatal administration of the SEB produced by *S. aureus* clearly augmented the proportion of $CD4^+$ T cells in the offspring spleen (20). Although lots of literatures reported that the SEB administration to animals, during adulthood, can affect the cytokine secretion (7-10), there is no information available about the influences of the prenatal SEB administration on cytokines in the offspring spleen. Therefore, the present study aims to explore the levels of the IFN-γ and the IL-4 in spleens of both neonatal and adult offspring whose mothers were administered intravenously with the SEB during pregnancy.

MATERIALS AND METHODS

Animals

The SD male and female rats were employed in the current investigation at three months of age. All rats were fed with a normal pellet diet and filtered tap water ad libitum and housed under temperature (22 to 25°C) and a light/dark cycle (12/12 h). Time-gated pregnant rats were acquired as our previous method (20). Ten pregnant rats at embryo day (ED) of 16 were separated into the PBS and the SEB groups in a random manner. Five rats were administered by vein once with 15 μg SEB (Sigma-Aldrich, St Louis, MO) as the SEB group. Others like the PBS group were treated with 0.3ml PBS in the same manner. Then, these animals were reared as above and permitted to normal labor. Spleens from neonates at 5 days in life and 3-month-old adult offspring were acquired under anesthesia with 10% chloralhydrate for the following study. In the present study, 24 neonates (two spleens from two neonates per experiment) and 12 adult offspring per group were used for all the experiments. The Animal Research Ethics Committee of Bengbu Medical College (Bengbu, permit No. 2011[146]) agreed with all the animal experimental procedures in the current study.

Quantitative Real-time PCR (qPCR)

TRIZOL reagent (Invitrogen Life Technologies, USA) was used to isolate the total RNA in neonatal or adult offspring spleens. A cDNA Synthesis Kit (Thermo Fisher Scientific Inc.) was applied to reversely transcribe the total RNA into the cDNA as

per the manufacturer's instructions. qPCR through a PCR instrument was employed to evaluate the gene expressions of the IFN-γ and the IL-4. The primers were used in the current study as Table 1. β-actin of housekeeping gene was employed as the internal control. The previous method (21) was chosen to calculate the mRNA level. Every independent experiment repeated three times was performed for reproducibility.

Western Blot

Western blot was applied for quantifying the level of the IFN-γ or the IL-4 protein. Briefly, the total proteins were extracted from the offspring spleen. Then, 10 μg protein was separated by the SDS-PAGE and transferred to the PVDF membrane. The solution of the IFN-γ and the IL-4 primary antibodies was incubated with the membrane overnight at 4°C. On the second day, the membrane was washed and incubated with the appropriate secondary antibody. Finally, the bound antibody was detected by the Chemiluminescence Kit (Millipore, USA). All samples were normalized to the β-actin signal as the control.

ELISA

The acquired spleen from each group was homogenized in the PBS. The supernatants of tissue homogenates were collected and a sandwich ELISA Kit (ABclonal Biotechnology, China) was applied to analyze the level of the IFN-γ or the IL-4. The detection limit of the IFN-γ and the IL-4 was 7.4 pg/ml and 7.5 pg/ml, respectively. Every separate experiment was treated in triplicates.

MeDIP-qPCR

The Methyl-DNA immunoprecipitation quantitative real-time PCR (MeDIP-qPCR) was employed to quantify the DNA methylation of the IFN-γ or the IL-4 in both neonatal and adulthood offspring spleens. The procedure was briefly as follows. The spleen was lysed with lysis buffer after it was broken in liquid nitrogen. To prepare genomic DNA, the lysed tissues were treated via proteinase K (Thermo Fisher Scientific, USA) and RNase digestion, phenol-chloroform extraction, and ethanol precipitation. After sonication, a part of the sonicated genomic DNA fragments was denatured and incubated with 5-methylcytosine monoclonal antibody overnight at 4 °C and the remains were left untreated to act as input control for the subsequent comparison with immunoprecipitated DNA. Following the incubation with mouse anti-IgG magnetic beads (BioLabs S1430S) for 2 h at 48 °C, magnetic bead complexes were sequentially washed at 4 °C using washing buffer. To set free the methylated DNA, these beads were resolved in 400 μl elution buffer and treated with 5mg/ml proteinase K before the methylated DNA was purified. Finally, the DNA methylation enrichment was determined by the qPCR using specific MeDIP primers as Table 2. qPCR amplification was treated as described above. TSH2B gene was used as the positive control and the GAPDH gene as the negative control.

Statistical Analysis

Statistical analysis was performed with the SPSS software using an independent T-test. The data normality was monitored with the

Table 1. Gene primers used in qPCR

Gene	Primer	Length(bp)
IL-4	F 5'-CATCGGCATTTTGAACGAGGTCA-3' R 5'-CTTATCGATGAATCCAGGCATCG-3'	240
INF-γ	F 5'-CATTGAAAGCCTAgGAAAGTCTG-3' R 5'-CTCATGAATGCATCCTTTTTCG-3'	267
β-actin	F 5'-CCCTAAGGCCAACCGTGAA-3' R 5'-CAGCCTGGATGGCTACGTACA-3'	83

Table 2. Used primers in this assay.

Gene	Primer	Length(bp)
IL-4	F:5' TGGTGGGAGGCAGTGAGTGT3' R:5' CTTGGCAGCAAGCACCTATTC3'	287
INF-γ	F:5' TGGGACCGTCTCATCGTCA3' R:5' GTGGCGGGAGCTTAGTTACTTT3'	110
TSH2B	F:5'TCGGCAAAGGGGACTACTATT3' R:5'CTCCTTGCGGCATCTCTTAC3'	99
GAPDH	F:5'GGACCCTGTGGTGCTTCATCT3' R:5'GGGCAGTAAGTGCTCCTAATCG3'	181

Kolmogorov-Smirnov test. The current data are presented as the mean ± SEM. A *p-value* of less than 0.05 was thought as statistically significant.

RESULTS

SEB Administration During Pregnancy Altered the Levels of the Splenic IFN-γ and the IL-4 in Neonatal Offspring

Fifteen micrograms of the SEB were administered by vein to the SD rats at ED16. To investigate the effect of the prenatal SEB administration on the cytokines IFN-γ or the IL-4 in neonatal offspring spleen, the spleen of the fifth-day neonatal offspring was harvested to determine the IFN-γ or the IL-4 cytokine with the ELISA, qPCR, and western blot. The results by the ELISA, and western blot suggested that the SEB administration, and during pregnancy, reduced the protein levels of the IFN-γ in addition to the IL-4 in the spleen of neonatal offspring compared with the PBS group (Figures 1A and C). Moreover, the analysis of qPCR revealed that the IFN-γ mRNA level in the SEB group was markedly augmented compared with the PBS group, and the IL-4 like the change of the IFN-γ (Figure 1B). Herein, the data from the relative amount of the IFN-γ or the IL-4 mRNA in the spleen tissue by qPCR were chosen to calculate the IFN-γ/IL-4 (22). The results indicated that the SEB administration, during pregnancy, augmented the ratio of the IFN-γ to the IL-4 (Figure 1D), suggesting that the SEB administration, during pregnancy, augmented more Th1 cytokine than the Th2 cytokine in neonatal offspring spleen.

SEB Administration, During Pregnancy, Augmented the Levels of Splenic IFN-γ and the IL-4 in Adult Offspring

To investigate the long-term effects of the SEB administration, during pregnancy, on cytokines IFN-γ as well as the IL-4, adult offspring spleen was harvested for determining the IFN-γ or the IL-4 level. As revealed by the ELISA method, prenatal SEB administration notedly increased the levels of the splenic IFN-γ as well as the IL-4 in adult offspring compared with the PBS group (Figure 2A). Moreover, the analysis of qPCR showed that the mRNA (Figure 2B) expressions of IFN-γ in addition to the IL-4 in the SEB group were augmented in contrast with the PBS group, and their proteins presented similar changes (Figure 2C). The results calculated via the data of the IFN-γ as well as the IL-4 mRNA indicated that the SEB administration, during pregnancy, increased the ratio of the IFN-γ to the IL-4 (Figure 2D), suggesting that the prenatal SEB administration inclined to increase the expression of the Th1 cytokine more than Th2 cytokine.

Prenatal SEB Administration Decreased the Methylation Levels of the IFN-γ as Well as the IL-4 in the Spleen of an Adult, Not Neonatal Offspring

To investigate the roles of epigenetic regulation by the prenatal SEB administration, the IFN-γ or the IL-4 gene methylation in the promoter regions was detected via MeDIP-

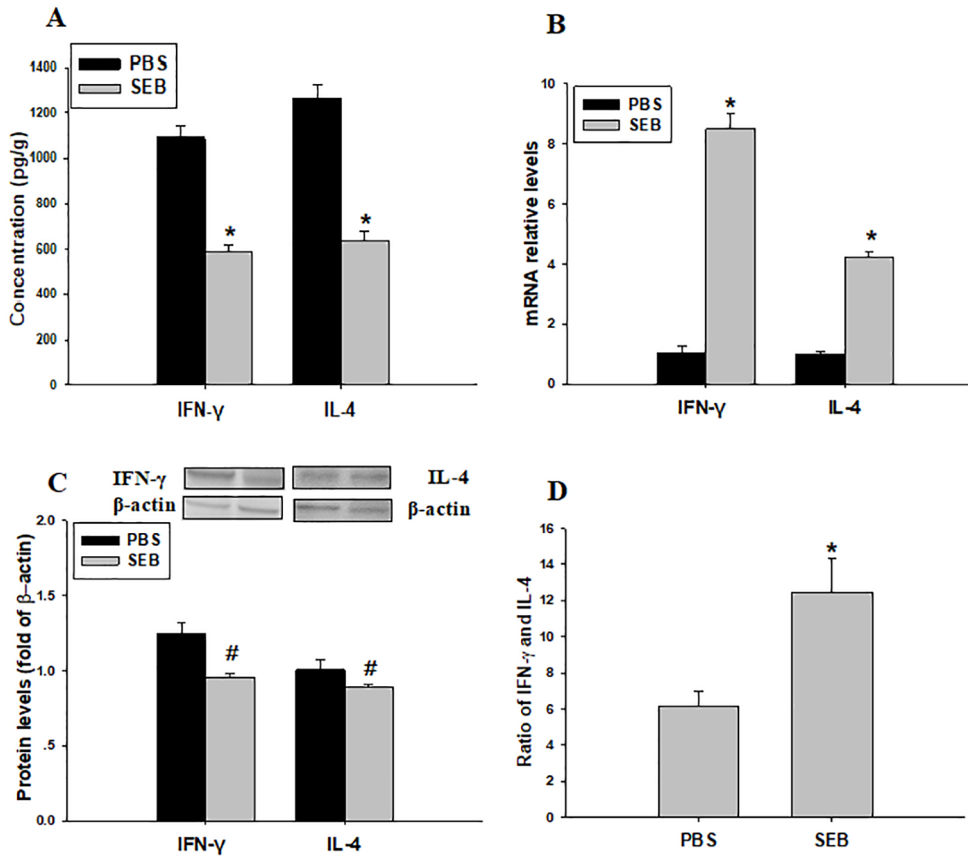

Figure 1. Effects of the SEB administration on pregnant rats, and on the IFN-γ or the IL-4 expression level in neonatal offspring spleen. The spleen was acquired from the fifth-day neonatal offspring in the two groups. The methods of the ELISA (A), qPCR (B), and western blot (C) were applied to determine the IFN-γ or the IL-4 levels. The results from the relative number of the IL-4 or the IFN-γ mRNA by qPCR were used to calculate their ratio (D). Every bar implies the mean±SEM from three separate experiments (two spleens from two neonates pool per assay) (#P<0.05; *P<0.01).

qPCR in the offspring spleen. As shown in Figures 3A and B, the SEB administration, and during pregnancy, exhibited no change in the methylations of the IFN-γ as well as the IL-4 genes in the neonatal offspring spleen. While in the spleen of adult offspring, the IFN-γ as well as the IL-4 gene methylations, remarkably reduced in the SEB group (Figures 3C and D). It was revealed that the prenatal SEB administration could modify the IFN-γ as well as the IL-4 expressions by an epigenetic change in the adult offspring spleen.

DISCUSSION

S. aureus can result in a serious infection in pregnant women during their pregnancy, which has become a promoting issue among pregnant women (16, 23). We have previously pointed out that the administration of the SEB produced by *S. aureus*, during pregnancy, significantly increased the percentage of $CD4^+$ T cells in the offspring spleen (20). But the influence of the SEB administration, and during pregnancy, on cytokines produced by $CD4^+$ T cells in offspring spleen is unclear. In the current research, first it is mentioned that the SEB administration, and during pregnancy, decreased the protein expression levels of the IFN-γ as well as the IL-4, but increased their mRNA levels and had no change in their methylation levels in the neonatal offspring spleen. However, in the adult offspring spleen, the SEB administration markedly increased the IFN-γ as well as the IL-4 expressions but decreased their methylation levels.

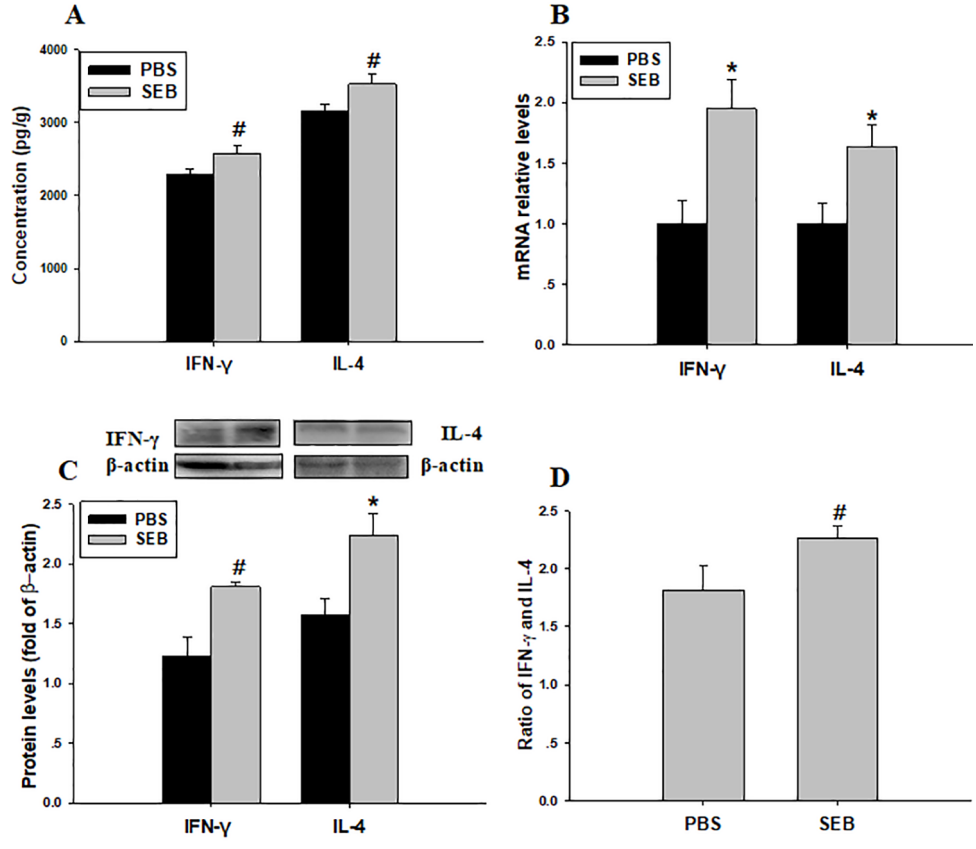

Figure 2. The SEB administration to pregnant rats increased the IFN-γ and the IL-4 expression levels in the adult offspring spleen. The spleen was acquired from 3-month-old adult offspring in the two groups. The methods of the ELISA (A), qPCR (B), and the western blot (C) were applied to determine the IFN-γ or the IL-4 levels. The results from the relative number of the IL-4 or the IFN-γ mRNA by qPCR were used to calculate their ratio (D). Every bar implies the mean±SEM from three separate experiments (a spleen was used for per assay) (#P<0.05; *P<0.01).

Given the antigen stimulation, $CD4^+$ T cells can be differentiated into distinct effector cell subsets of the Th1 as well as the Th2 which produced the characteristic cytokine hallmark. The Th1 type cell mainly yields the IFN-γ and the IL-2, but the Th2 produces the IL-4, IL-10, and the IL-13 (11, 12). In the present study as others (24, 25), the IFN-γ and IL-4 are commonly shown as the representative cytokines of these two cells. A line of studies have revealed that the SEB administration could induce high levels of various inflammatory effectors, for instance, the IFN-γ and the IL-4, which can lead to lethal shock in animal models (2, 26). But the influence of the prenatal SEB administration on the expression of the IFN-γ as well as the IL-4 is unknown. This research has indicated that the SEB administration to pregnant rats not only markedly reduced the IFN-γ protein level but also the IL-4 level in neonatal offspring spleen, whereas it notedly increased their expression levels in the adult offspring spleen. This finding has revealed that the SEB administration to pregnant rats caused a mixed response of the Th1 as well as the Th2 cells in offspring spleen, in line with other studies on the effect of the SEB on the pathogenesis of atopic dermatitis (27).

The paradigm of the Th1 and the Th2 provides a framework for understanding the T cell biology. The imbalance between the Th1 and the Th2 cytokines contributes to autoimmune (28), allergic (29), and chronic inflammatory diseases (30). Although prenatal administration of the SEB could elicit mixed Th1 and Th2 cytokines, this finding instantly has posed a scientific question: whether or not

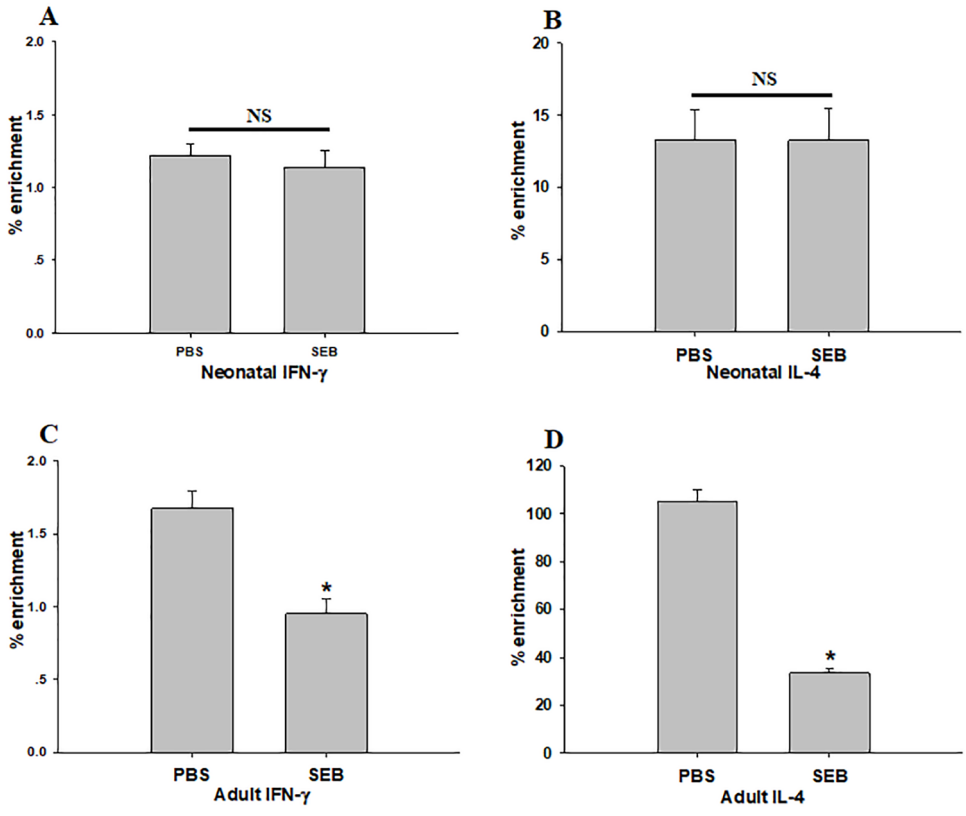

Figure 3. The SEB administration to pregnant rats reduced the IFN-γ and the IL-4 gene methylation levels in the spleen of an adult, not neonatal offspring. The spleen was acquired from the fifth-day neonatal and the adult offspring and was applied to determine the IFN-γ or the IL-4 gene methylation by MeDIP-qPCR. (A) IFN-γ gene methylation in neonates. (B) IL-4 gene methylation in neonates. (C) IFN-γ gene methylation in adult offspring. (D) IL-4 gene methylation in adult offspring. Every bar implies the mean±SEM from three separate experiments (a spleen from an adult or two spleens from neonates was used for per experiment) (*P<0.01; NS, no significance).

the prenatal SEB administration has a bias on the Th1 or the Th2 cytokines. To address this question, we have focused on the ratio of the IFN-γ to the IL-4. All data, which was calculated via relative amounts of the IL-4 or the IFN-γ mRNA in the spleen, showed that the SEB administration to pregnant rats induced the increased ratio of the IFN-γ to IL-4 in offspring spleen, indicating that the SEB administration, and during pregnancy, resulted in the imbalance of the Th1 and the Th2 cells and caused cytokine expression change by skewing toward the Th1 cells response. This biased Th1 cytokines profile has a harmful influence on the embryo development and may indicate a pathologic pregnancy (31, 32) because a dominant Th2 cytokines profile is related to the normal pregnancy (33).

During fetal development, the pregnant stimulus of many harmful factors can cause lasting structural and functional influences and produce imprinting diseases (34, 35). We have previously revealed that the SEB administration to pregnant rats could program a similar increase of the $CD4^+$ T cells from neonates to adult rats (20). The current study was interesting since it discovered that the prenatal SEB administration resulted in reduced protein expression of splenic the IFN-γ as well as the IL-4 in neonatal offspring, instead of their increases in the adult offspring. This phenomenon of reversibility is consistent with the effects of prenatal dexamethasone or virus administration on the immune and neurological systems of the offspring because the embryo or neonate is more sensitive to immunotoxic modulation than the adult (36, 37).

Prenatal administration of physicochemical and biological factors can yield epigenetic

modification, which presents a significant regulator of disease susceptibility. Gene methylation is one of many common epigenetic modifications, which modifies gene expression to transmit across generations to mediate the effect of the adverse environmental stimulus on the offspring health (38, 39). The DNA methylation in the promoter region blocks the binding of protein at these methylated sites, and it may inhibit gene transcription (40). In the present study, the analysis of MeDIP-qPCR showed that the SEB administration to pregnant rats notedly reduced the IFN-γ as well as the IL-4 methylation levels in the adult offspring spleen, in agreement with their increased expression levels. These data revealed that the SEB administration, and during pregnancy, could regulate the IFN-γ as well as the IL-4 expression by the epigenetic change in the adult offspring spleen. It is noteworthy that no changes were indicated in the methylation levels of the IFN-γ as well as the IL-4 in neonatal offspring spleen, although their mRNA expressions clearly increased and their protein expressions notedly decreased. These data revealed that the IFN-γ and the IL-4 methylation changes induced by the SEB administration, during pregnancy, might exhibit a delayed effect to transmit across generations. Herein, the prenatal SEB administration caused the contrary changes between mRNA and protein expressions of the IFN-γ or the IL-4 in neonatal offspring spleen. There may exist a post-transcriptional regulation (41), but its mechanism needs further studies.

CONCLUSION

According to the findings of this study, the SEB administration to pregnant rats markedly decreased the IFN-γ and the IL-4 methylation levels, and augmented their mRNA and protein expression levels in the adult offspring spleen, although the SEB administration had no effect on the IFN-γ as well as the IL-4 methylation levels in neonatal offspring spleen. In conclusion, according to the existing research, it is believed that the SEB administration to pregnant rats at ED16 alters Th1 and Th2 cytokine expressions via decreasing the methylation in the spleen of the adult but, not the neonatal offspring. The current study does, however, have a few limitations. Firstly, the whole spleen which was not sorted for $CD4^+$ T cells was applied to analyze the IFN-γ as well as the IL-4 expression levels because these cytokines in the spleen are mainly produced by the $CD4^+$ T cells. Secondly, although the IFN-γ or the IL-4 can be used as the produced characteristic cytokine of the Th1 or the Th2 cells, other cytokines produced by the Th1 and the Th2 cells should be considered in the future studies.

AUTHOR CONTRIBUTIONS

Jun-chang Guan planned and designed the experiments. Jing Sun, Jia-bao Zhao, and Chao Sun performed the experiments. Ping Zhou analyzed the data. Yu-ting Zhu and Shu-xian Gao revised the manuscript. Ying-ao Fan, Hao-yuan Jiang, and Qing-Wei Zheng helped to experiment. Jun-chang Guan wrote the manuscript. All authors read and approved the manuscript.

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Conflicts of Interest: None declared.

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