JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Gao *et al.*, *Journal of Medical Microbiology* 2020;69:591–599
DOI 10.1099/jmm.0.001152

MICROBIOLOGY SOCIETY

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Prenatal exposure of staphylococcal enterotoxin B attenuates the development and function of blood regulatory T cells to repeated staphylococcal enterotoxin B exposure in adult offspring rats

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Abstract

Introduction. Staphylococcal enterotoxin B (SEB) is an extensively studied super-antigen. A previous study by us suggested that SEB exposure during pregnancy could alter the percentage of $CD4^+$ and $CD8^+$ T cells in the peripheral blood of neonatal offspring rats.

Aim. It is unknown whether SEB exposure during pregnancy can influence the development of regulatory T cells (Tregs) in the peripheral blood of neonatal offspring rats.

Methodology. Pregnant rats at gestational day 16 were intravenously injected with 15 µg SEB. Peripheral blood was acquired from neonatal offspring rats on days 1, 3 and 5 after delivery and from adult offspring rats for determination of Treg number by cytometry, cytokines by ELISA, and FoxP3 expression by real-time PCR and western blot.

Results. SEB given to pregnant rats significantly increased the absolute number of Tregs and the expression levels of FoxP3, IL-10 and TGF-β (P<0.05, P<0.01) in the peripheral blood of not only neonatal but also adult offspring rats. Furthermore, repeated SEB exposure in adult offspring rats significantly decreased the absolute number of Tregs (P<0.01), and the expression levels of FoxP3, IL-10 and TGF-β (P<0.05, P<0.01) in their peripheral blood.

Conclusion. Prenatal SEB exposure attenuates the development and function of Tregs to repeated SEB exposure in the peripheral blood of adult offspring rats.

INTRODUCTION

Staphylococcus aureus is one of the most common bacterial pathogens of hospital and community-acquired infections, ranging from skin infections to severe toxic shock syndrome [1]. Staphylococcal enterotoxins produced by *Staphylococcus aureus* have several serotypes such as A, B, C1, C2, C3, D, E, G, H and I [2]. Staphylococcal enterotoxin B (SEB) has been extensively studied as a super-antigen (SAg). Several studies have shown that SAg exposure to an individual induced the activation of a large fraction of both $CD4^+$ and $CD8^+$ T cells, mounting specific Vβ chains in their T cell receptors. Subsequently, the majority of the expanding T cells would be deleted by induction of apoptosis and the remaining SAg-reactive T cells would be anergic because of no proliferation with *in vitro* SEB re-exposure [3, 4].

The rat is an important model organism to help understand human infectious diseases because it is closely related to humans in terms of anatomy, physiology and genetics [5–7]. The rat immune system as in humans includes immune organs, lymphocytes and cytokines. As a subset of T lymphocytes, T regulatory cells (Tregs) in line with their origin are classified as either thymic-derived/natural Tregs (nTregs) or peripheral/inducible Tregs (iTregs) [8]. The forkhead transcription factor Foxp3 is currently considered as the most selective marker for

Received 10 September 2019; Accepted 09 January 2020; Published 11 February 2020
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Keywords: staphylococcal enterotoxin B; peripheral blood; Tregs; pregnancy; offspring.
Abbreviations: DOHaD, developmental origin of health and disease; FBS, fetal bovine serum; GD, gestation day; IL-10, interleukin-10; PBS, phosphate-buffered saline; SAg, super-antigen; SEB, staphylococcal enterotoxin B; TGF-β, transforming growth factor-β; Tregs, regulatory T cells.
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Tregs [9]. Tregs that can suppress the activation and function of other T cells of the immune system play important roles not only in maintaining tolerance to self-antigen but also in preventing an extended inflammatory response to infection [10, 11]. The ability of Tregs to suppress immune responses mainly involves direct cell–cell contact in a process that is dependent on signalling via cytotoxic T-lymphocyte antigen 4, as well as secretion of transforming growth factor-β (TGF-β) and IL-10 ([12]. Several studies [13, 14] have revealed that SEB exposure can induce development of the anergic T cell population as Tregs that can suppress the primary response of SAg-specific T cells both *in vivo* and *in vitro*.

The developmental origin of health and disease (DOHaD) hypothesis postulates that early life exposures of adverse environmental stimuli can influence disease outcomes throughout the whole lifespan of an organism [15]. A large amount of epidemiological evidence from humans and animal experimental studies has suggested an association between adverse stimuli during pregnancy and some adult diseases [16, 17]. *Staphylococcus aureus* is an important factor leading to adverse pregnancy outcomes and fetal development abnormalities in pregnant women [18, 19]. A previous study by us [20] suggested that SEB exposure during pregnancy could alter the percentage of $CD4^+$ and $CD8^+$ T cells in the peripheral blood of neonatal offspring rats. Because Tregs function in modulating the activation and function of other T cells to suppress immune responses and the suppressive capacity of Tregs is required to maintain the balance of T helper cells [21], we were interested in seeing whether SEB exposure during pregnancy can influence the development of Tregs in the peripheral blood of not only neonatal but also adult offspring rats.

METHODS

Animals

Thirty adult female Sprague–Dawley rats were used for the present study. The rats were reared with rodent chow and filtered tap water provided *ad libitum* on a 12 h light/dark cycle at 25 °C. Pregnant rats were acquired as previously described [20]. Briefly, each female rat was mated with a male rat and checked each morning for the presence of a vaginal plug. Day 1 of gestation (GD) was defined as the day when a plug was initially observed in the vagina. Time-gated pregnant rats at GD 16 were randomly divided into a PBS group and an SEB group (15 pregnant rats in each group). In the SEB group, 0.3 ml of 50 µg ml^{-1} SEB (Sigma-Aldrich) in 0.2 M PBS was administrated to the pregnant rats injected once via the tail vein [22]. The pregnant rats in the PBS group were intravenously injected once with the same volume of PBS. These pregnant rats were allowed to deliver naturally. One group of the neonatal offspring rats at days 1, 3 and 5 (30 neonates at day 1, 30 neonates at day 3, 15 neonates at day 5) after delivery were killed to obtain peripheral blood for the following experiments, while the other group were reared to adulthood (about 3 months). Some of the adult offspring rats (n=15) were used to give peripheral blood from abdominal aorta for the following experiments, while the rest (n=15) were used for the study of *in vivo* response to repeated SEB exposure. All animal experiments in this study were approved by the Animal Research Ethics Committee of Bengbu Medical College (Bengbu, permit No.2011[146]).

In vivo response of the adult offspring rats to repeated SEB exposure

The adult offspring rats born to mothers injected with PBS or SEB during pregnancy were treated with either PBS or SEB, separately, as for the pregnant rats. The groups were named respectively as PBS+PBS, PBS+SEB, SEB+PBS and SEB+SEB. Five days after administration, the peripheral blood of adult offspring rats in each group was taken for the following analysis.

Preparation of cell suspensions in periphery blood

Red Blood Cell Lysis Buffer (Beyotime Institute of Biotechnology) was used to remove red blood cells from the whole blood of both neonatal and adult offspring rats as described previously [20]. The white pellet was suspended in PBS containing 2% fetal bovine serum (FBS) (HyClone). One part of the suspended cells was used for western blot and real-time PCR, while the other part was counted in a Burker-Turk haemocytometer (Emergo) using a Leica DM500 microscope, and 10^6 cells were stained for analysis by flow cytometry.

Flow cytometry

For surface staining, single cell suspensions obtained as above were stained for 30 min on ice with the following fluorescence conjugated antibodies including FITC-conjugated anti-CD3, APC-conjugated anti-CD4 and PE-conjugated anti-CD25 from eBioscience. For intracellular staining, cell suspensions after surface staining were fixed and permeabilized using the Intracellular Fixation and Permeabilization Buffer Set (eBioscience) as per the manufacturer's instructions. Then the fixed/permeabilized cells were stained with Percp-Cy5.5-conjugated anti-Foxp3 (eBioscience) for 45 min on ice. The stained cells were analysed by flow cytometry on a FACS calibur (Becton Dickinson) using CellQuest analysis software (BD Biosciences).

ELISA

The peripheral blood obtained from the neonatal and adult offspring rats as above was isolated for plasma by centrifugation. Expression levels of TGF-β and IL-10 in the plasma were analysed by ELISA kits (ABclonal) according to the manufacturer's instructions. Each independent experiment was performed in triplicate.

Real-time PCR

TRIzol (Invitrogen) reagent was used to extract total RNA from the suspended cells obtained as described above and total RNA was reverse transcribed into cDNA using a cDNA Synthesis Kit (Thermo Fisher Scientific). An SYBR Green PCR kit (Qiagen) was used to quantify Foxp3 mRNA levels

Table 1. Used primer sequence

Gene	Primer sequence
FoxP3	F:5′-GGCTCTACTCTGCACCTTCC-3′ R:5′-GCAGTGGGTAGGATCCTTGT-3′
β-Actin	F:5′-CCCTAAGGCCAACCGTGAA-3′ R:5′-CAGCCTGGATGGCTACGTACA-3′

by real-time PCR performed with a PCR instrument (Applied biosystems. *β*-Actin was used as an internal control. PCR primers (Table 1) for Foxp3 and *β*-actin were designed based on published genomic sequences in GenBank and synthesized by Sangon Biotech.

Western blot

The cells obtained as described above were resuspended in 150 µl of cell protein lysis buffer. Total protein was quantified using Bradford assays and 80 µg was analysed by western blotting with anti-Foxp3 or anti-*β*-actin (Abcam) monoclonal antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Millipore), then exposed to X-ray film. Photographs were digitized and protein expression was normalized to *β*-actin. The magnitude of the immune signal was determined as a percentage of the internal control.

Statistical analysis

An independent *t*-test was used to assess the significance of Tregs, cytokines, as well as mRNA and protein levels of Foxp3 in the periphery blood of neonatal and adult offspring rats. To assess the differences of the above in the peripheral blood of adult offspring rats responding to repeated PBS and SEB exposure, Turkey's-b in one-way ANOVA was used. Statistical significance was defined as $P<0.05$.

RESULTS

Effects of SEB-primed exposure to pregnant rats on Tregs in the peripheral blood of offspring rats

Several studies [13, 14] have shown that SEB exposure can induce the development of the anergic T cell population as Tregs. We therefore wanted to investigate if prenatal SEB exposure can affect Treg development in the peripheral blood of offspring rats. Flow cytometry revealed that SEB exposure to pregnant rats in the SEB group significantly increased the absolute numbers of Tregs in the peripheral blood of neonatal offspring rats on days 1, 3 and 5 after delivery compared with the PBS group, although Treg percentages were not different (Fig. 1a–b). To investigate whether this change in neonates remains to adulthood, Treg numbers in adult offspring rats were also determined. Absolute numbers of Tregs in the SEB group were clearly higher than that in the PBS group in the peripheral blood of adult offspring rats (Fig. 1c–d). These results suggest that prenatal SEB exposure could significantly influence Treg development in offspring rats. We did not differentiate between the sexes because our previous study [23] found that the effects of SEB exposure to pregnant rats during pregnancy on T cells were not different between female and male offspring rats.

Effects of SEB-primed exposure to pregnant rats on FoxP3 expression in the peripheral blood of offspring rats

Expression of FoxP3, as the most specific marker for Tregs [24], was also determined by real-time PCR and western blot. SEB-primed exposure to pregnant rats significantly increased the expression levels of FoxP3 mRNA (Fig. 2a) and protein (Fig. 2b) in the peripheral blood of neonatal offspring rats on day 5 after delivery. Expression levels of FoxP3 mRNA (Fig. 2c) and protein (Fig. 2d) in the peripheral blood of adult offspring rats showed similar changes to neonatal offspring rats in the SEB group compared with the PBS group. These data suggest that prenatal SEB exposure could significantly influence FoxP3 expression, which is consistent with the change in Treg numbers.

Effects of SEB-primed exposure to pregnant rats on cytokine levels in the peripheral blood of offspring rats

To assess the suppressive function of Tregs [25, 26], the cytokines IL-10 and TGF-*β* were investigated. SEB-primed exposure to pregnant rats significantly increased the levels of IL-10 (Fig. 3a) and TGF-*β* (Fig. 3b) detected by ELISA in the peripheral blood of neonatal offspring rats on days 1, 3 and 5 after delivery. In the peripheral blood of adult offspring rats, expression levels of IL-10 (Fig. 3c) and TGF-*β* (Fig. 3d) showed similar changes to neonatal offspring rats between the SEB and PBS groups.

Effects of repeated SEB exposure on Tregs in the peripheral blood of adult offspring rats born to SEB-primed pregnant rats

To assess the response of Tregs to repeated *in vivo* SEB exposure, the adult offspring rats were re-injected with SEB and Treg numbers were determined in peripheral blood. The absolute numbers of Tregs increased significantly in the PBS+SEB group compared with the PBS+PBS group, while the absolute numbers of Tregs in the SEB+SEB group were significantly lower than those in the PBS+SEB or SEB+PBS groups (Fig. 4b). These data suggest that repeated *in vivo* SEB exposure could attenuate the development response of Tregs in the peripheral blood of adult offspring rats born to mothers exposed prenatally to SEB.

Effects of repeated SEB exposure on FoxP3 expression in the peripheral blood of adult offspring rats born to SEB-primed pregnant rats

As above, the effects of repeated SEB exposure on FoxP3 expression were also assessed. Compared with the PBS+PBS group, the expression levels of FoxP3 mRNA (Fig. 5a) or protein (Fig. 5b) in the peripheral blood increased significantly in the PBS+SEB group, while the expression levels of FoxP3 mRNA or protein were markedly lower in the SEB+SEB

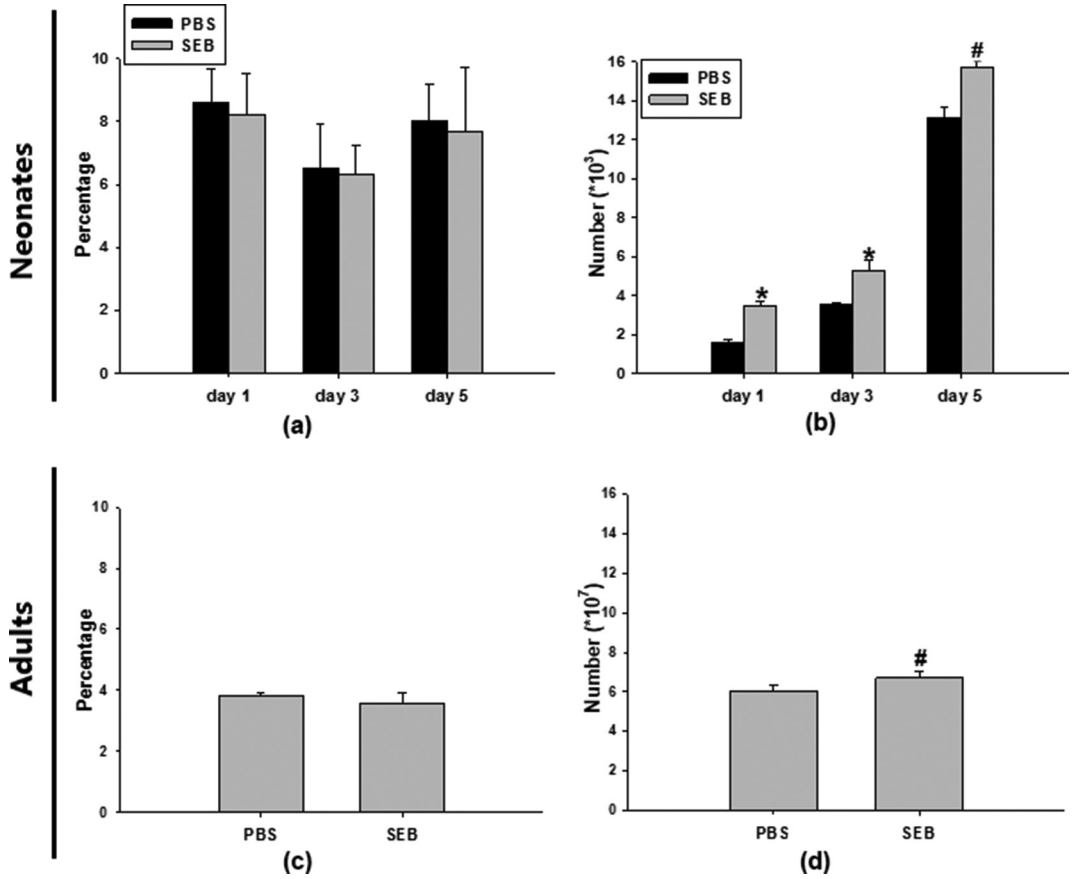

Fig. 1. Effect of prenatal SEB exposure on Tregs in the peripheral blood of offspring rats. Percentages and numbers of Tregs were determined by flow cytometry in peripheral blood of neonatal offspring rats on days 1, 3 and 5 after delivery and adult offspring rats (about 3 months) in both PBS and SEB groups. (a) Neonatal Treg percentages; (b) neonatal Treg numbers; (c) adult Treg percentages; and (d) adult Treg absolute numbers. Values were calculated with data from 15 independent experiments. Data represent mean±SE. Compared with the PBS group: $^{\#}P<0.05$; $^{*}P<0.01$.

group than those in the PBS+SEB or SEB+PBS groups. These data suggest that *in vivo* repeated SEB exposure could also decrease expression of FoxP3 as a specific marker for Tregs in the peripheral blood of adult offspring rats born to mothers exposed prenatally to SEB.

Effects of repeated SEB exposure on cytokines in the peripheral blood of adult offspring rats born to SEB-primed pregnant rats

Because repeated *in vivo* SEB exposure attenuated Treg development, we further investigated whether repeated SEB exposure could affect Treg function via cytokines. Levels of IL-10 (Fig. 6a) and TGF-β (Fig. 6b) in peripheral blood increased significantly in the PBS+SEB group compared with the PBS+PBS group, while their levels were significantly lower in the SEB+SEB group than those in the PBS+SEB and SEB+PBS groups. These data suggest that repeated *in vivo* SEB exposure could decrease the function of Tregs in adult offspring rats born to mothers exposed prenatally to SEB.

DISCUSSION

In the immune system of an individual to maintain tolerance to SAg and prevent an extended inflammatory response to infection, Tregs play important roles via suppressing the activation and function of other T cells [21]. A previous study by us [20] suggested that SEB exposure to pregnant rats could alter the percentage of $CD4^+$ and $CD8^+$ T cells in the peripheral blood of offspring rats, but it was unknown whether SEB exposure to rats during pregnancy influences the development of Tregs in the peripheral blood of offspring rats. In the present study, we show for the first time that SEB exposure to pregnant rats can significantly increase the absolute number of Tregs and the expression levels of FoxP3, IL-10 and TGF-β in the peripheral blood not only of neonatal but also of adult offspring rats. Furthermore, repeated SEB exposure to adult offspring rats significantly decreased the absolute number of Tregs, and the expression levels of FoxP3, IL-10 and TGF-β in their peripheral blood.

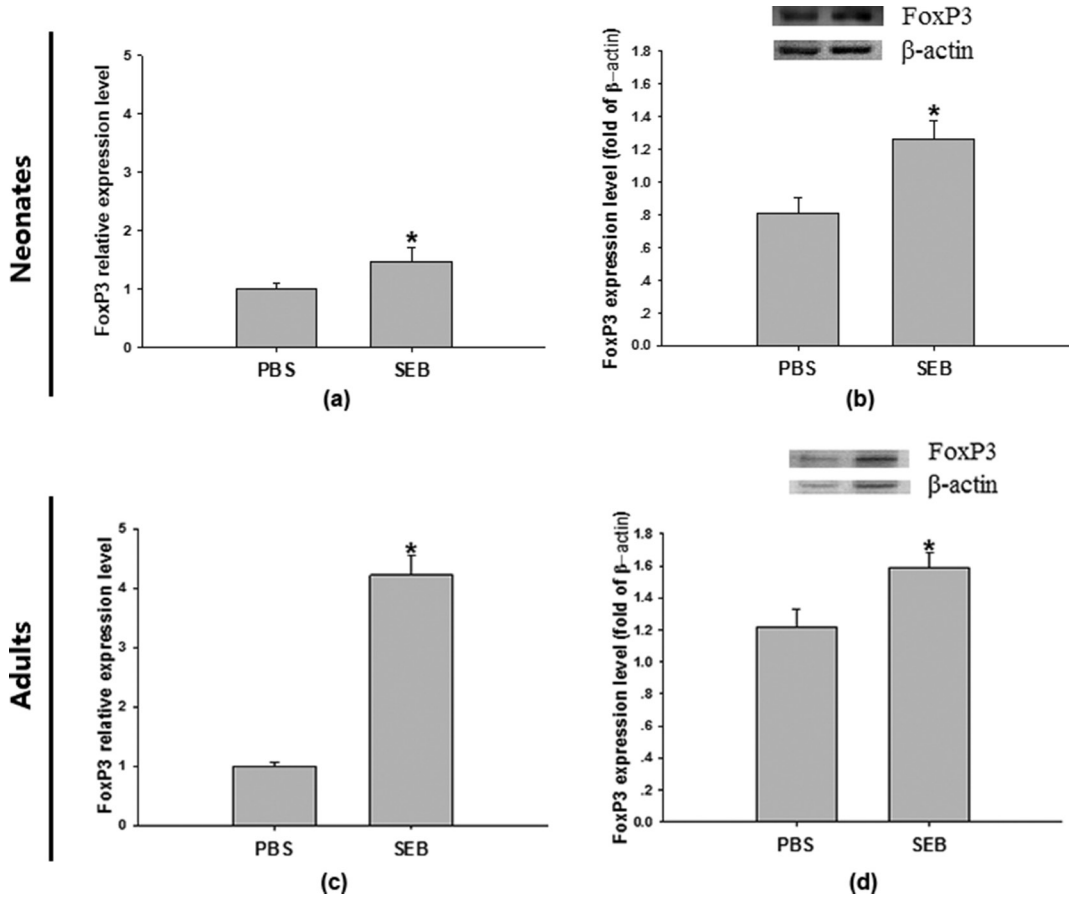

Fig. 2. Effects of prenatal SEB exposure on FoxP3 expression in the peripheral blood of offspring rats. FoxP3 expression level was determined by real-time PCR and western blot in peripheral blood of neonatal offspring rats on day 5 after delivery and adult offspring rats (about 3 months) in both PBS and SEB groups. (a) Neonatal FoxP3 mRNA level; (b) neonatal FoxP3 protein level; (c) adult FoxP3 mRNA level; and (d) adult FoxP3 protein level. Values are represented based on data from three independent experiments. Data represent mean±SE. Compared with the PBS group: **P*<0.01.

Tregs include thymic-derived/natural Tregs (nTregs) and peripheral/inducible Tregs (iTregs) according to their origin [8]. Although nTregs express the glucocorticoid-induced TNF receptor and CTLA-4 at high levels [27, 28], similar to CD25 both of these molecular markers have limitations to distinguish nTregs from iTregs in that they are expressed by activated conventional $CD4^+CD25^-$ T cells as well. To date, there is a lack of specific markers to distinguish between these two cell populations. FoxP3 is commonly considered to be the most specific marker for Tregs [24]. The cell markers CD4, CD25 and FoxP3 were therefore chosen for the current study to mark Tregs via flow cytometry. A plethora of studies in adult animals showed that *in vivo* exposure of peripheral T cells to bacterial SAgs caused partial deletion of SAg-reactive CD4 T cells. SAg-reactive T cells that were not deleted would be heterogeneous, containing both anergic cells and Tregs [29]. The present study is the first to study the effect of SEB exposure during pregnancy on Treg development in the peripheral blood of offspring rats. We found that SEB exposure to pregnant rats significantly increased the absolute number of Tregs and the expression levels of FoxP3 in the peripheral blood of neonatal offspring rats on days 1, 3 and 5 after delivery, but no change in Treg percentages. The immune organ thymus is fundamental to the establishment and renewal of the peripheral T-cell compartment with a diverse repertoire. nTregs develop in the thymus during the early stages of fetal T-cell development [30]. Some nTregs can migrate from the thymus to the circulation and constitute 5–10% of peripheral $CD4^+$ T cells in mice [31]. It has also been reported that bacterial SAgs can induce expansion of human Tregs ($CD4^+CD25^+Foxp3^+$ cells) efficiently from $CD4^+CD25^-Foxp3^-$ T cells in peripheral blood [32]. Thus, the increased Tregs in the present study might be due to recruitment of nTregs from the thymus, conversion of $Foxp3^-$ to $Foxp3^+$ T cells, and/or proliferation of natural or converted $Foxp3^+$ T cells.

Since Sakaguchi [33] first found Tregs with regulatory capacity, numerous studies have reported the roles of Tregs in establishing self-tolerance and preventing an excessive response to infection [10, 11]. The mechanism mediating these roles for Tregs mainly pass through direct cell–cell contact dependent on secretion of the cytokines TGF-β and IL-10

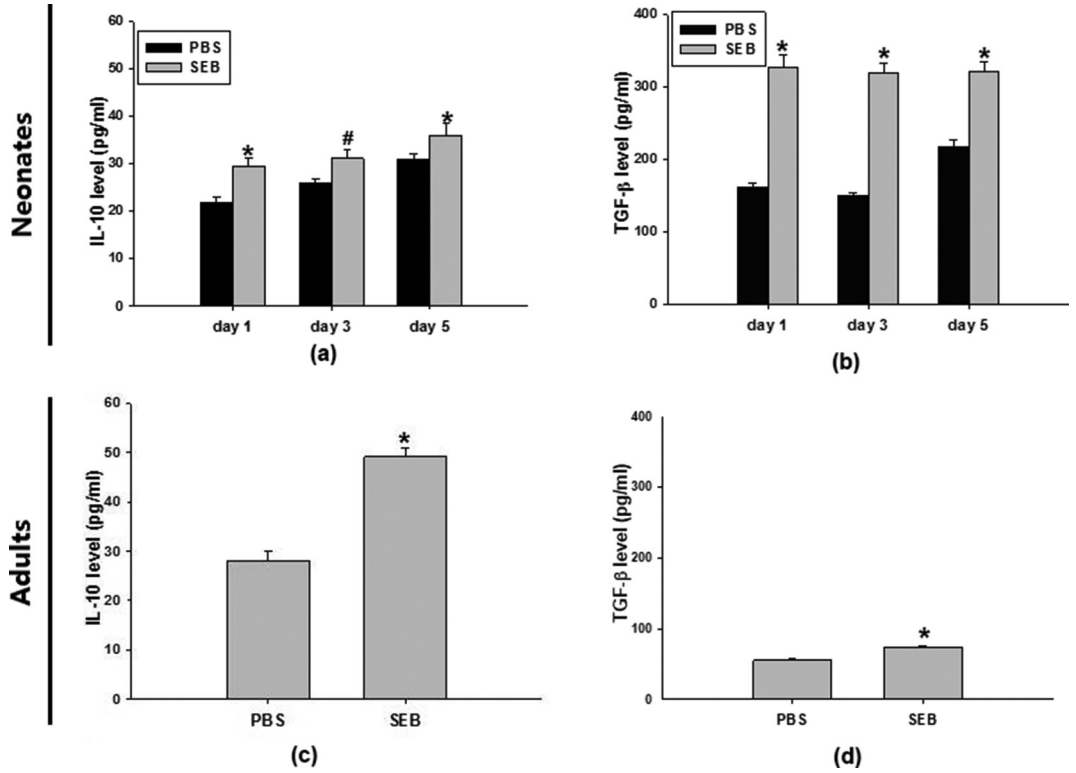

Fig. 3. Effects of prenatal SEB exposure on cytokines in the peripheral blood of offspring rats. Cytokines were tested by ELISA in peripheral blood of neonatal offspring rats on day 5 after delivery and adult offspring rats (about 3 months) in both PBS and SEB groups. (a) Neonatal IL-10 level; (b) neonatal TGF-β level; (c) adult IL-10 level; and (d) adult TGF-β level. Values were calculated based on data from ten independent experiments. Data represent mean±SE. Compared with the PBS group: $^{\#}P<0.05$; $^{*}P<0.01$.

[12]. Several previous studies have associated the suppressive function of Tregs with IL-10 and TGF-β [25, 26]. Expression of Foxp3, as a specific marker for Tregs, is also required for $CD4^{+}CD25^{+}$ Tregs to fulfil their regulatory function [24, 34]. Several studies have suggested that stimulation of SAgs could induce the expression of FoxP3 in a dose-dependent manner as well as the production of IL-10 and TGF-β [32, 35] for functional suppression. Consistent with the above studies was the observation in the present study that SEB exposure to pregnant rats significantly increased the expression levels of FoxP3 mRNA and protein, as well IL-10 and TGF-β in the peripheral blood of neonatal offspring rats. This suggested that SEB exposure during pregnancy might increase the suppressive and regulatory ability of Tregs in the peripheral

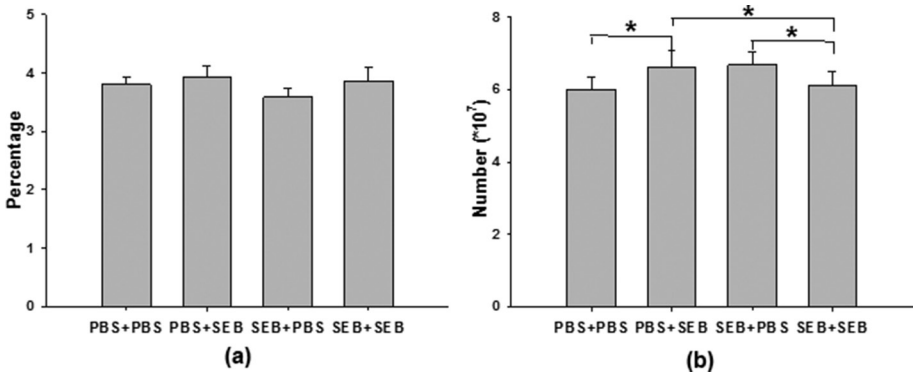

Fig. 4. Effects of repeated SEB exposure on Tregs in the peripheral blood of adult offspring rats. Adult offspring rats of mothers injected with PBS or SEB during pregnancy were re-administrated with either PBS or SEB, separately, and 5 days later the peripheral blood of adult offspring rats was used for the analysis of Treg percentages (a) and absolute number (b). Values were calculated based on data from 15 independent experiments. Data represent mean±SE. $^{*}P<0.01$.

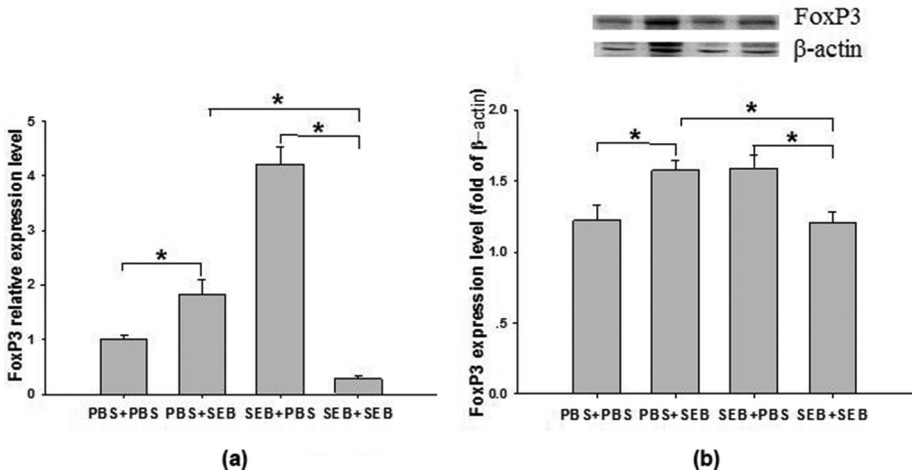

Fig. 5. Effects of repeated SEB exposure on FoxP3 expression in the peripheral blood of adult offspring rats. Five days after adult offspring rats were exposed to either PBS or SEB, their peripheral blood was acquried for analysis of expression levels of (a) FoxP3 mRNA by real-time PCR and (b) protein by western blot. Values were calculated based on data from three independent experiments. Data represent mean±se. **P*<0.01.

blood of neonatal offspring rats within the immune system. The increased ability may help the offspring rats to establish tolerance to SEB later in life, although this tolerance might be harmful for their survival.

Pregnant adverse stimuli, including physicochemical and biological factors during fetal development, may lead to long-term structural and functional effects and form programming/imprinting effects to cause adult diseases [16, 17]. In a murine model, the addition of microbes to pregnant mice increased the prevalence of certain groups of intestinal innate immune system cells through altering haematopoietic cell development in offspring [36]. The current study found that SEB exposure to pregnant rats could significantly increase the absolute number of Tregs as well as the expression levels of FoxP3, IL-10 and TGF-β in the peripheral blood of adult offspring rats, similar to the changes seen in neonatal offspring rats. These data suggested that prenatal SEB exposure could imprint the changes of Tregs from the neonatal stage to adulthood. A previous study showed that repeated *in vivo* SEB exposure to induce tolerance required the presence of Tregs [13]. It raised the question of whether prenatal SEB exposure influences the *in vivo* response of Tregs to repeated SEB administration in the peripheral blood of adult offspring rats. The results from the present study show that repeated SEB exposure to adult offspring rats which were exposed prenatally to SEB significantly decreased the absolute number of Tregs and the expression levels of FoxP3, IL-10 and TGF-β in their peripheral blood, in contrast to the changes in these indexes in adult offspring rats exposed prenatally to PBS. These data therefore suggest that prenatal SEB exposure could attenuate the development and function of Tregs to repeated SEB exposure in the peripheral blood and this change might cause adverse effects in adult offspring rats.

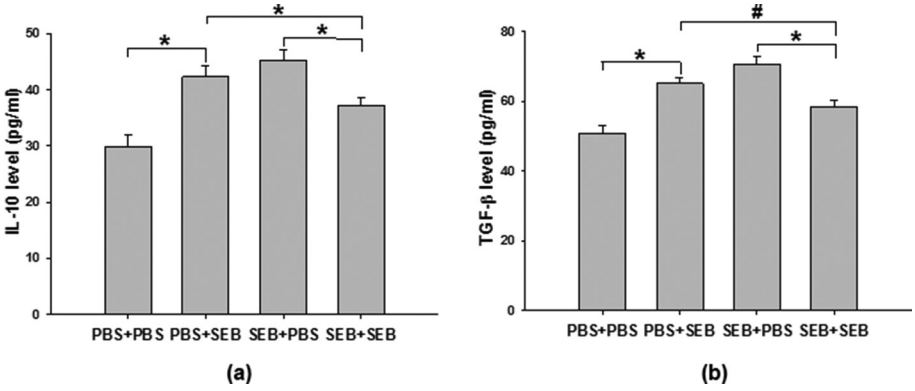

Fig. 6. Effects of repeated SEB exposure on cytokines in the peripheral blood of adult offspring rats. Five days after adult offspring rats were were exposed to either PBS or SEB, their peripheral blood was used for the analysis of (a) IL-10 and (b) TGF-β expression levels. Values were calculated based on data from ten independent experiments. Data represent mean±SE. #*P*<0.05; **P*<0.01.

In conclusion, SEB exposure to pregnant rats could significantly increase the absolute number of Tregs and the expression levels of FoxP3, IL-10 and TGF-β in the peripheral blood of not only neonatal but also adult offspring rats. These findings revealed that SEB exposure during pregnancy could imprint these changes of Treg indexes in offspring rats and might increase the suppressive and regulatory ability of Tregs in the peripheral blood of offspring rats within the immune system. Furthermore, repeated SEB exposure to adult offspring rats could attenuate the development and function of Tregs to repeated SEB exposure in the peripheral blood and this change might cause adverse effects in adult offspring rats.

Funding information
This work was supported by grants from the National Natural Science Foundation of China (81571454) and the Scientific Research Foundation of Bengbu Medical College (BYKF1812).

Conflicts of interest
The authors declare that there are no conflicts of interests.

Ethical statement
All animal experiments in this study were approved by the Animal Research Ethics Committee of Bengbu Medical College (Bengbu, permit No.2011[146]).

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