Staphylococcal enterotoxin B administration during pregnancy imprints the increased CD4:CD8 T-cell ratio in the peripheral blood from neonatal to adult offspring rats

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INTRODUCTION

Staphylococcal enterotoxin B (SEB) is an important superantigen (SAg), which has been extensively investigated in five groups (A through E) of staphylococcal enterotoxin (Bergdoll, 1983). SEB can cross-link major histocompatibility complex class II molecules with a variable portion of the β chain (Vβ) of the T cell receptor, binding beyond the antigen-specific site, which does not require classical antigen-processing and presentation, and can polyclonally stimulate T cells (Bell & Buxser, 1995). A line of studies (MacDonald et al., 1991; Seth et al., 1994) have shown that the immune response to SAg is biphasic; an initial activation phase characterized by T-cell proliferation is followed by a period of anergy and/or tolerance owing to deletion of the appropriate Vβ-expressing T cells by apoptosis. Our previous study (Yang et al., 2014) demonstrated that SEB administration during pregnancy could alter the percentage of the T-cell subpopulation in the thymus of the neonatal rats and SEB restimulation in an in vitro cultured thymus further decreased the percentage of Vβ8.2 T cells, but little is known about the effect of maternal SEB administration during pregnancy on T-cell subpopulations in the peripheral blood of the offspring rats.

Fetal programming has become an increasingly accepted concept; it suggests that an adverse environmental stimulus experienced during a critical period of fetal development may induce long-term structural and functional effects in the developing organism (Barker, 1994). A body of epidemiological evidence in humans and animal experimental models indicate an association between pregnant adverse stimulus and some adult diseases (Ruchat et al., 3 These authors contributed equally to this work. Abbreviations: FBS, fetal bovine serum; GD, gestational day; SAg, superantigen; SEB, staphylococcal enterotoxin B.
2013; Vickers, 2014; Kilcoyne et al., 2014; Krow-Luca et al., 2014). Microbial infection is a common stimulus to fetal development during pregnancy, so whether SEB administration during pregnancy can program the alteration of T-cell subpopulations in the offspring rats is another question to be elucidated.

Therefore, in the present study, pregnant rats at gestational day (GD) 16 were intravenously injected with SEB. The percentages of CD4 and CD8 T cells were determined in the peripheral blood of both neonatal and adult offspring rats. The effect of SEB administration during pregnancy on the in vivo response of T cells to secondary SEB administration was also investigated in the peripheral blood of the adult offspring rats.

**METHODS**

**Animals.** Three-month-old Sprague–Dawley rats were employed in this study and housed in a controlled environment of 22 to 25 °C and a 12 h light/dark cycle, with rodent chow and filtered tap water provided ad libitum. Each female rat was mated with a male rat and checked each morning for the presence of a vaginal plug. Day 1 of gestation was defined as the day when a plug was initially observed in the vagina. When the pregnancy was confirmed, the females were isolated from the males and kept in separate cages. Time-gated pregnant rats were randomly divided into the following two groups at GD 16: the PBS (control) group and the SEB group. In the SEB group, the pregnant rats were intravenously injected once with 0.3 ml SEB (Sigma-Aldrich; prepared as 50 μg SEB ml⁻¹ in 0.2 M PBS). The pregnant rats in PBS group were intravenously injected once with the same volume of PBS. They were allowed to give birth naturally. The peripheral blood of the neonatal offspring rats between day 0 and 5 after delivery was acquired by truncation for the following experiments.

For the experiments with adult offspring rats, the neonatal rats born to the mothers were kept with their mothers until weaning. At weaning, the male and female offspring rats were separated and reared to adulthood (about 3 to 5 months). Then, the adult offspring rats from the above two groups were intravenously injected with either SEB or PBS, separately, in the same manner as the pregnant rats. Five days after administration, the peripheral blood of the adult offspring rats was harvested from abdominal aorta for the following experiments. The adult offspring rats were intravenously injected once with 0.3 ml SEB (Sigma-Aldrich; prepared as 50 μg SEB ml⁻¹ in 0.2 M PBS). The pregnant rats in PBS group were intravenously injected once with the same volume of PBS. They were allowed to give birth naturally. The peripheral blood of the neonatal offspring rats between day 0 and 5 after delivery was acquired by truncation for the following experiments.

**Preparation of cell suspensions in the periphery blood.** For the removal of red blood cells from whole blood, the peripheral blood of both the neonatal and adult offspring rats was treated with Red Blood Cell Lysis Buffer (Beyotime Institute of Biotechnology) according to manufacturer’s instructions. After removing the supernatant, a white pelleted was collected in balanced PBS solution supplemented with 2 % heat-inactivated fetal bovine serum (PBS; HyClone). The cells were washed three times by centrifugation (400 g for 10 min at 4 °C), resuspended in staining buffer (PBS containing 2 % FBS and 0.02 % NaN3). Nucleated cells were counted in a Burker–Turk haemocytometer (Emergeo) using a microscope (Leica DMS500; Leica) and 10⁶ cells were stained for flow cytometry analysis, as described below.

**Flow cytometric analysis.** Single cell suspensions obtained as above were stained using directly conjugated mAbs specific for CD3, CD4 and CD8 (eBioscience) at 4 °C for 30 min, washed three times with PBS, and then fixed with 1 % paraformaldehyde. The labelled cells were then analysed by flow cytometry on a FACSCalibur (Becton Dickinson) using CellQuest analysis software (BD Biosciences). Dead cells were excluded on the basis of low forward-light scatter.

**ELISA analysis.** Expression levels of IL-4 and IFN-γ in the plasma of neonatal and adult offspring rats were assayed by ELISA (R&D systems) according to the manufacturer’s instructions. Standard curves were determined for IL-4 for a range of 2–600 pg ml⁻¹ and IFN-γ for a range of 5–1000 ng ml⁻¹. The OD₄₅₀ was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek). ELISAs were performed in triplicate. The concentrations of the samples were calculated by fitting the OD₄₅₀ values of each sample into the equation generated from the standard curve graph.

**Statistical analysis.** To assess the significance of T cells and cytokines difference in the peripheral blood, Tukey’s B in one-way ANOVA was used. Independent Student’s t-test was used to assess the differences in cytokines and T cells in the periphery blood of adult offspring rats restimulated with PBS and SEB. Statistical significance was defined as P<0.05.

**RESULTS**

**Effect of prenatal SEB exposure on CD4 and CD8 T cells in the peripheral blood of neonatal rats**

Pregnant rats at GD 16 were intravenously administrated 15 μg SEB and the peripheral blood of the neonatal rats between day 0 to 5 after delivery was acquired to determine the percentages of CD4 and CD8 T cells. The percentage of CD4 T cells in the SEB group was significantly higher than in the PBS group in the peripheral blood of the neonatal rats between day 0 to 5 after delivery (Fig. 1a). There was no difference in the percentage of CD8 T cells between the PBS and SEB groups in the peripheral blood of the neonatal rats between day 0 to 4 after delivery, but on day 5 after delivery, the percentage of CD8 T cells in the SEB group was significantly lower than in the PBS group (Fig. 1b). Furthermore, the ratio of CD4 to CD8 T cells in the peripheral blood of the neonatal rats between day 0 to 5 after delivery was also analysed. The results in Fig. 1(c) showed the ratio of CD4 to CD8 T cells in the SEB group was significantly higher than that in the PBS group in the peripheral blood of the neonatal rats between day 0 to 5 after delivery. The ratios that increased gradually in two groups in the peripheral blood of the neonatal rats between day 0 to 5 after delivery were less than one from day 0 to 1 and more than one from day 2 to 5.

**Effect of the prenatal SEB exposure on CD4 and CD8 T cells in the peripheral blood of the adult offspring rats**

Pregnant rats at GD 16 were intravenously administrated 15 μg SEB and the neonatal offspring rats were reared to adulthood. Then, the peripheral blood of the adult offspring rats was acquired to determine the percentages of CD4 and CD8 T cells. It was found that the percentage of CD4 T cells in the SEB group was significantly higher than that in the PBS group in the peripheral blood of the adult offspring rats (Fig. 2a), while the prenatal exposure of
SEB significantly reduced the percentage of CD8 T cells in the peripheral blood of the adult offspring rats (Fig. 2b). Furthermore, the ratio of CD4 to CD8 T cells in the SEB group was significantly higher than in the PBS group in the peripheral blood of the adult offspring rats (Fig. 2c).

Effect of secondary SEB administration on CD4 and CD8 T cells in the peripheral blood of the adult offspring rats exposed prenatally to SEB

The adult offspring rats in both PBS and SEB groups were intravenously injected with either SEB or PBS, separately. Five days after administration, the peripheral blood of the adult female and male offspring rats was harvested from abdominal aorta for the determination of the percentages of CD4 and CD8 T cells. The results in from the PBS group in Fig. 3 showed that SEB administration significantly increased the percentages of CD4 T cells and significantly decreased the percentages of CD8 T cells in the adult female and male offspring rats compared with those of PBS administration. While in the SEB group, the secondary SEB administration significantly decreased the percentages of CD4 T cells and significantly increased the percentages of CD8 T cells in the adult female and male offspring rats compared with those of PBS administration.

Quantitative expression of IL-4 and IFN-γ in plasma

In the plasma of the neonatal rats 3 days after delivery, the expression levels of IL-4 (Fig. 4a) and IFN-γ (Fig. 4b) in the SEB group were significantly higher than those in the PBS group. After the neonatal offspring rats were reared to adulthood, the expression levels of IL-4 (Fig. 4c) and IFN-γ (Fig. 4d) in the plasma exhibited a similar change to those in the neonatal rats. When the adult offspring rats in both the PBS and SEB groups were intravenously injected with SEB, compared with the rats with PBS, the expression levels of IL-4 (Fig. 4c) and IFN-γ (Fig. 4d) in the plasma were significantly decreased.
DISCUSSION

In the present study, the results showed that the prenatal exposure of SEB significantly altered the percentages of both CD4 (CD4 T cells detected in the present study is a subpopulation of T cells and includes CD4, CD25, Foxp3 and T regulatory cells) and CD8 T cells, as well as the ratio of CD4 to CD8 T cells in the peripheral blood of both neonatal and adult offspring rats. Secondary SEB administration significantly decreased the percentage of CD4 T cells and increased the percentage of CD8 T cells in the adult offspring rats exposed prenatally to SEB. To the best of our knowledge, this is the first study to link the prenatal SEB exposure to changes in T-cell subpopulations in the peripheral blood of offspring rats.

Injection of SEB into naive mice induced specific tolerance associated with a selective deletion of peripheral V$\beta$8 T cells (Rellahan et al., 1990; Kawabe & Ochi, 1991; Goettelfinger et al., 2000; Li et al., 2008). This deletion of T cells by SEB appeared at both central and peripheral immune compartments (Webb et al., 1990; MacDonald et al., 1991). Our previous studies (Guan et al., 2012; Yang et al., 2014) demonstrated the effect of SEB administration during pregnancy on the central compartment (thymus), but the effect of maternal SEB administration during pregnancy on the peripheral compartment is largely unknown. The present study revealed that, in the peripheral blood of the neonatal offspring rats, the prenatal exposure of SEB could induce a decrease in CD8 T cells on the fifth day after delivery and the increase of CD4 T cells between day 0 to 5 after delivery. While in the peripheral blood of the adult offspring rats, the prenatal exposure of SEB could also induce decreased CD8 T cells and increased CD4 T cells, which is consistent with other results from direct SEB administration in adult mice (MacDonald et al., 1991; Baschieri et al., 1993). The prenatal exposure of SEB also resulted in increased expression levels of IL-4 (Th2 type cytokine) and IFN-$\gamma$ (Th1 type cytokine) in the plasma of neonatal and adult offspring rats, consistent with the change of CD4 T cells affected by SEB. These data suggest that prenatal exposure to SEB induced a decrease in the percentage of CD8 T cells accompanied by a relative increase in the percentage of CD4 T cells, which is similar to the change of CD4 and CD8 thymocyte levels in the thymus of the offspring rats observed previously (Yang et al., 2014). This finding immediately raised an important question: why did the prenatal exposure of SEB have no alteration on the percentage of CD8 T cells in the

![Fig. 2. Effect of the prenatal SEB exposure on CD4/CD8 T cells in the peripheral blood of the adult offspring rats. The peripheral blood of the adult offspring rats was harvested from abdominal aorta in both the PBS and SEB groups. The percentages of both CD4 (a) and CD8 (b) T cells were analysed by flow cytometry and the ratio of CD4 to CD8 T cells (c) was calculated from the percentages. Values were calculated with data from ten independent experiments. Each experiment included a male/female adult offspring rat. Data represent mean±s.e. Compared with the PBS group: *P<0.05; **P<0.01.](image-url)
peripheral blood of the neonatal rats between day 0 to 4 after delivery? To address this question, we focused on the ratio of CD4 to CD8 T cells. The data showed that the ratio of CD4 to CD8 T cells was less than one in the peripheral blood of the neonatal rats between day 0 to 1 after delivery, and was more than one from day 2 after delivery to adulthood, which is consistent with other results (Kelly & Scollay, 1992; Jiménez et al., 2001). As a consequence, no

**Fig. 3.** Effect of secondary SEB administration on CD4/CD8 T cells in the peripheral blood of the adult offspring rats exposed prenatally to SEB. The adult offspring rats in both the PBS and SEB groups were intravenously injected with either SEB (named as PBS + SEB and SEB + SEB) or PBS (named as PBS + PBS and SEB + PBS), separately. 5 days after administration, the peripheral blood of the adult female and male offspring rats was harvested from abdominal aorta. The percentages of both CD4 (a) and CD8 (b) T cells were analysed by flow cytometry. Values were calculated with data from ten independent experiments. Each experiment included a male/female adult offspring rat. Data represent mean ± SE. Compared with PBS + PBS: *P < 0.05; compared with SEB + PBS: **P < 0.05.

**Fig. 4.** Quantitative expression of IL-4 and IFN-γ in plasma. The plasma was acquired from the peripheral blood of both the neonatal and adult offspring rats. The expression levels of IL-4 and IFN-γ in the plasma of both the neonatal (a, b) and adult (c, d) offspring rats were assayed by ELISA kits. Values were calculated with data from ten independent experiments. Data represent mean ± SE. Compared with the PBS group: *P < 0.01. Compared with PBS + PBS: **P < 0.05; compared with PBS + PBS or SEB + PBS: #P < 0.05.
alteration about the percentage of CD8 T cells in the peripheral blood of the neonatal rats between day 0 to 4 after delivery could be necessary for the inversion of the CD4:CD8 T-cell ratio. This is an interesting question that requires further study.

Pregnant adverse stimulus including physico-chemical and biological factors during fetal development may induce long-term structural and functional effects and yield programming/imprinting effects to cause diseases (Kaplan et al., 2011; Ruchat et al., 2013; Cordero et al., 2013; Vickers, 2014; Kilcoyne et al., 2014; Krow-Lucal et al., 2014). The present study revealed that prenatal exposure to SEB significantly increases the CD4:CD8 T-cell ratio through the decrease of CD8 T cells and the increase of CD4 T cells in the peripheral blood of both neonatal and adult offspring rats. Owing to the inability of SEB to cross the placenta, these results suggested that the effect of the prenatal SEB exposure on T cells was indirect and was able to imprint the increased CD4:CD8 T-cell ratio from the neonate to adulthood. Furthermore, whether the prenatal exposure to SEB influenced the in vivo response of CD4 and CD8 T cells to SEB restimulation in the peripheral blood of the adult offspring rats was also investigated. The data from the present study showed that SEB administration during adulthood significantly increased the percentage of CD4 T cells and decreased the percentage of CD8 T cells in the peripheral blood of the adult offspring rats exposed prenatally to PBS. On the contrary, adult offspring rats exposed prenatally to SEB followed by secondary SEB administration during adulthood significantly decreased their percentage of CD4 T cells and increased their percentage of CD8 T cells. These data suggested that prenatal exposure to SEB altered the response characteristics of CD4 and CD8 T cells to secondary SEB administration in the peripheral blood and this change may cause adverse effects in the adult offspring rats.

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