

## Research Paper

**Predominant Th2-type response during normal pregnancy of rats**ZHANG Qing-Hong<sup>1,\*</sup>, HUANG Yan-Hong<sup>2</sup>, HU Yu-Zhen<sup>1</sup>, WEI Geng-Ze<sup>1</sup>, LU Shun-Yan<sup>1</sup>, ZHAO Yu-Feng<sup>1</sup><sup>1</sup>Department of Physiology, Institute of Basic Medical Science, and <sup>2</sup>Department of Gynaecology and Obstetrics, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, China

**Abstract:** The immunological parameters were analyzed during pregnancy of Lewis rats by the methods of flow cytometry, thymidine incorporation and enzyme-linked immunospot (ELISPOT). MHC II of spleen mononuclear cells (MNCs) and CD11c of periphery blood MNCs was apparently downregulated in late pregnancy, while the costimulatory molecules B7-1 and B7-2 showed no difference. Increased expression of Th2 cytokines (IL-10, IL-4) and TGF $\beta$  was detected in the spleen and peripheral blood MNCs in the third trimester by flow cytometry. No suppression of Th1 cytokine represented by IFN $\gamma$  was found. Furthermore, antigen specific proliferation of spleen and peripheral blood MNCs was unchanged, but higher proliferation of MNCs from mesenteric lymph nodes was shown in late pregnancy. There was an inhibition of antigen specific antibody production in pregnancy examined by ELISPOT. These data indicate the immunomodulatory effects of sex-hormones in pregnancy, which may be related to the remission of T cell-mediated autoimmune diseases during pregnancy.

**Key words:** sex-hormone; immune system; autoimmune disease; pregnancy; rat

**正常妊娠大鼠的 Th2 型免疫反应**张庆红<sup>1,\*</sup>, 黄艳红<sup>2</sup>, 胡玉珍<sup>1</sup>, 韦耿泽<sup>1</sup>, 吕顺艳<sup>1</sup>, 赵玉峰<sup>1</sup>第四军医大学<sup>1</sup>基础部生理教研室, <sup>2</sup>西京医院妇产科, 西安 710032

**摘要:** 采用流式细胞仪, <sup>3</sup>H-TdR 掺入和酶联免疫打点(enzyme-linked immunospot, ELISPOT)方法, 研究妊娠免疫学指标的改变。妊娠晚期大鼠脾脏单个核细胞表面分子主要组织相容性抗原 II (MHC II) 明显下调, 外周血单个核细胞表达 CD11c 明显减少, 共激活分子 B7-1 和 B7-2 未见改变; 脾脏和外周血单个核细胞中 Th2 细胞因子 IL-10、IL-4 表达增多, TGF $\beta$  阳性细胞数也明显增加, 而 Th1 细胞因子 IFN $\gamma$  的产生未受抑制。此外, 脾脏和外周血中单个核细胞的抗原特异性增殖未见改变, 而腹腔淋巴结细胞的增殖明显升高。脾脏单个核细胞在妊娠晚期分泌较少的抗原特异性抗体。提示妊娠期激素具有免疫调节作用, 可能与怀孕时 Th1 细胞介导的自身免疫性疾病得到缓解有关。

**关键词:** 性激素; 免疫系统; 自身免疫性疾病; 妊娠; 大鼠

**中图分类号:** Q492.6; R392.6

It has been well established that multiple sclerosis (MS) and other putative Th1 cell-mediated autoimmune diseases improve clinically during pregnancy<sup>[1]</sup>, but the mechanisms have not been fully elucidated. Multiple changes in the immune system occur during human pregnancy, which have been globally characterized as suppression of cell-mediated (Th1 or Th2) immunity and stimulation of humoral immunity. It has been hypothesized that these changes are necessary to prevent immunological rejection of the allogeneic fetus<sup>[2]</sup>. The study of human immune system in

pregnancy is limited on periphery blood mononuclear cells (MNCs) because human spleen and lymph nodes are not available at any time. So far, little attention has been paid on the immune variations in rat pregnancy, especially on the parameters related to autoimmune diseases. We carried out the study on pregnant Lewis rats to achieve a systemic understanding of immune response in pregnancy.

Considerable research has been dedicated to investigate the existence and potential of anergy in immune regulation. The tolerance in pregnancy may imply the

Received 2003-07-16 Accepted 2003-08-25

This work was supported by innovation project (No. CX02A024) from the Fourth Military Medical University.

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changes of the surface markers on MNCs, resulting anergy of T cells. To testify our hypothesis, several surface markers on MNCs were detected by flow cytometry.

To function in adaptive immunity, the rare antigen-specific lymphocytes must proliferate extensively before they differentiate into functional effector cells. Thus, the analysis of induced lymphocyte proliferation is a central issue in our study. It was shown that a modest decrease in proliferation was consistently observed in draining lymph nodes (DLN) isolated from estrogen-treated mice<sup>[3]</sup>. We considered if it might also apply to pregnant rats and contributed to the remission of Th1-mediated disease.

It has long been known that an increased intrathecal production of immunoglobulin (Ig) in cerebrospinal fluid (CSF) is observed in more than 90% of multiple sclerosis (MS) patients<sup>[4]</sup>. It's not clear if the Ig production is decreased in pregnant patients, thus contributing to the disease remission.

In this study, we investigated the immunological parameters in rat pregnancy in order to elucidate the mechanism for the remission of the Th1-mediated autoimmune disease in pregnancy.

## 1 MATERIALS AND METHODS

**1.1 Reagents.** Guinea pig myelin basic protein (MBP) covering amine acid residues 68~86 (MBP<sub>68-86</sub>; YGSLPQKSQRSQDENPV) was synthesized in an automatic Tecan/Syro synthesizer (Multisyntech, Bochum, Germany), concanavalin A (Con A), acetylcholine receptor (AChR) and ovalbumin (OVA) were purchased from Sigma. Monoclonal anti-rat CD11c monoclonal antibody (mAb), TGF $\beta$  mAb, phycoerythrin (PE)-conjugated anti-rat MHC II, IL-10 mAb, FITC-conjugated anti-rat IFN $\gamma$  mAb were purchased from Serotec. Mouse anti-rat B7-1 and B7-2 mAbs, PE-conjugated anti-rat IL-4 mAb were obtained from PharMingen.

**1.2 Animals.** Female Lewis rats, 6~8 weeks old, were provided by the experimental animal center of our university.

**1.3 Preparation of blood MNCs.** On day 25 after crossing with male rats, the female rats were entered into the experiments as late pregnancy (third trimester). Peripheral blood was obtained from the heart by exposing the chest. MNCs suspensions were prepared by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). Spleen was removed under aseptic condition. MNCs suspensions were obtained by grinding the organs through a stainless steel wire mesh. Erythrocytes were osmotically lysed. Cells were then resuspended in DMEM medium

(Gibco), supplemented with 1% MEM amino acids (Life Technologies), 2 mmol/L glutamine (Gibco), 10 nmol/L HEPES (Sigma), and 10% heat-inactivated fetal calf serum (FCS, Life Technologies). All the cells were then adjusted to  $2 \times 10^9$  cells/L.

**1.4 Flow cytometry.** For cell surface staining,  $1 \times 10^5$  cells were incubated with mouse anti-rat CD11c, B7-1 or B7-2 for 30 min, followed by PE-conjugated anti-mouse secondary Ab. PE-conjugated anti-rat MHC II were detected directly after incubating with MNCs for 30 min. All procedures were performed in 1% BSA in PBS. For intracellular cytokine staining,  $2 \times 10^5$  cells were fixed with 2% formaldehyde, permeabilized with 0.5% saponin (Sigma), and then incubated with FITC-conjugated anti-rat IFN $\gamma$  or PE-conjugated anti-rat IL-4, IL-10. Unlabeled TGF $\beta$  were stained for 30 min, followed by PE-conjugated anti-mouse secondary Ab. All procedures were performed in 0.5% saponin/1% BSA in 0.01 mol/L PBS. Ten thousand cells were analyzed by a FACScan flow cytometer (Becton Dickinson).

**1.5 Lymphocyte proliferation assays.** Proliferative responses of MNCs were examined by <sup>3</sup>H-TdR incorporation. Briefly, 200  $\mu$ l of MNCs suspensions ( $2 \times 10^9$  cells/L) were incubated in 96 well polystyrene microtiter plates (Nunc) at 37°C in 5% CO<sub>2</sub> with or without MBP<sub>68-86</sub> (10 mg/L), AChR (5 mg/L) or OVA (5 mg/L) for 48 h. Cells were pulsed with <sup>3</sup>H-Thymidine (1.85 MBq/L per well) for 12 h before harvested and <sup>3</sup>H-TdR incorporation was measured in a liquid beta scintillation counter.

**1.6 Analysis of anti-MBP<sub>68-86</sub>, AChR, ConA antibody by enzyme-linked immunospot (ELISPOT).** ELISPOT assay was adopted for detection of antibody-secretion at the single-cell level. Nitrocellulose bottom microtiter plates (Millititer-HAM plates, UK) were coated with 100  $\mu$ l aliquots of antigens (10 mg/L). MNCs suspensions ( $5 \times 10^8$  cells/L) were added to individual wells. After 48 h of culture, the wells were extensively washed. Then the plates were incubated with 100  $\mu$ l of polyclonal rabbit anti-rat IgG (1:400, Innogenetics) for 24 h at 4°C. After washing, the plates were incubated with biotinylated swine anti-rabbit IgG (1:500, Vector Laboratories) and then with avidin-biotin peroxidase complex (1:200, Vector) followed by peroxidase staining. The red-brown immunospots, which corresponded to the cells that had secreted antigen specific antibodies were counted under a dissection microscope.

**1.7 Statistic analysis.** Differences between two groups were tested by Student's *t* test. The level of significance was set at 0.05.

## 2 RESULTS

### 2.1 Changes in surface marker on MNCs

Several surface markers such as MHC II, CD11c and costimulating molecules B7-1, B7-2 were detected. It was shown that MHC II was remarkably downregulated in spleen in pregnancy, while CD11c was reduced in blood MNCs (Fig. 1). Costimulating molecules B7-1, B7-2 of

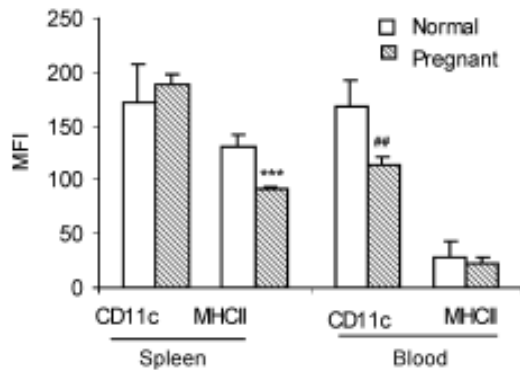


Fig. 1. Phenotypical characteristics of spleen and blood MNCs from rats in the third trimester of pregnancy. Mean fluorescence intensity of the surface markers was detected by flow cytometry. The differences are compared using the *t* test for paired samples. Data are means $\pm$ SD, *n*=6. \*\*\**P*<0.001, ##*P*<0.01 vs normal virgin rats.

MNCs were not altered in pregnant rats (data not shown).

### 2.2 Percentage of Th1, Th2 and TGF $\beta$ -expressing cells in the peripheral blood and spleen MNCs in later pregnancy

The percentages of Th2 cells represented by IL-10 and IL-4 in blood and spleen MNCs were significantly higher in pregnant rats, while TGF $\beta$ -expressing cells expanded

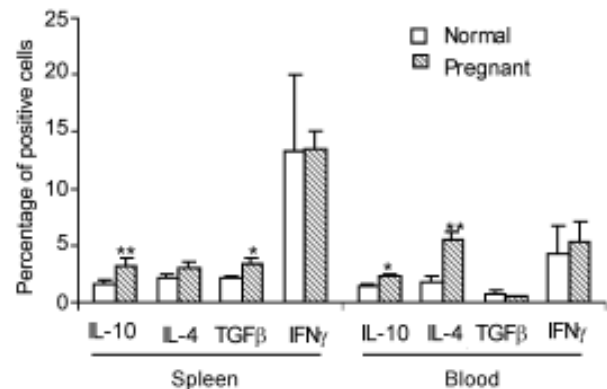


Fig. 2. Th2 cytokine predominant expression in spleen and blood MNC in the third trimester of pregnancy. MNCs were freshly separated and examined by flow cytometry immediately. The percentages represent the incidences of positive cells. MNCs from pregnant rats and virgin rats were analysed in paired cultures, and differences are compared using the *t* test for paired samples. *n*=6. \**P*<0.05, \*\**P*<0.01 vs normal.

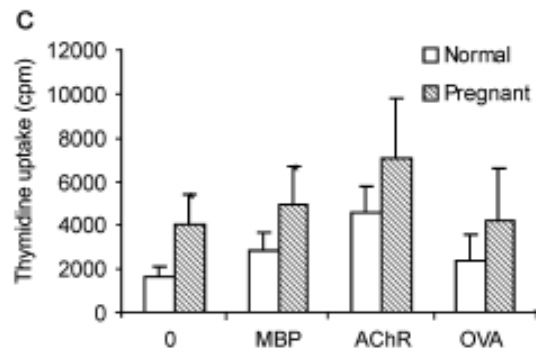
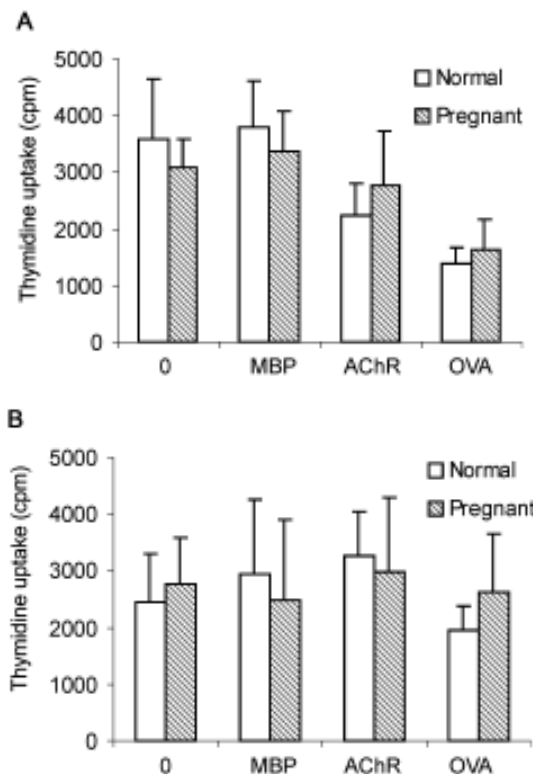


Fig. 3. Proliferation of MNCs from spleen (A), blood (B) and lymph nodes (C) in the third trimester of pregnancy. MNCs were cultured with MBP, AChR and OVA respectively for 48 h. Increased proliferation of MNCs from pregnant rats detected by  $^3\text{H}$ -TdR incorporation was only found in lymph nodes.

only in spleen MNCs. The percentage of Th1 cells represented by IFN $\gamma$  remained the same in pregnant rats (Fig.2).

### 2.3 Ag specific proliferation of MNCs from blood, spleen and lymph nodes

As to MNCs from both spleen (Fig.3A) and blood (Fig. 3B) of pregnant rats, they proliferated to the similar extent with those from virgins in the presence of several autoantigens. In contrast, MNCs from the mesenteric lymph nodes in pregnant rats showed significantly augmented proliferation compared to virgin rats (Fig.3C). In late pregnancy, although lack of statistical significance, the lymph node MNCs stimulated with autoantigens exhibited a trend of higher proliferating ability than those cultured in pure medium (Fig.3C).

### 2.4 Decreased anti-AChR and anti-OVA antibody in pregnant rats

An increase in humoral immunity has been reported during pregnancy and during high-dose estrogen therapy. Consequently, the humoral immune response to respective autoantigens in pregnancy was carried out in our study. Considerable amount of anti-AChR and anti-OVA antibodies could be detected, but only the production of anti-AChR antibody was significantly decreased in pregnant rats along with a decreasing tendency of anti-OVA antibody production. Unexpectedly, not so many MBP-specific antibodies were observed in this experiment (Fig. 4).

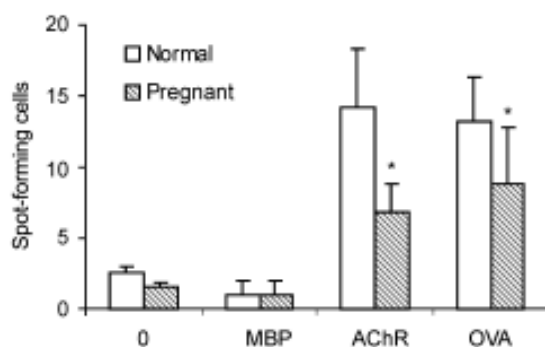


Fig. 4. Decreased AChR or OVA-specific antibody production of spleen MNCs in the pregnant rats. Nitrocellulose plates were coated with antigens overnight at 4°C, then spleen MNCs were cultured for another 48 h. Spot-forming cells were stained brown under a dissection microscope.

## 3 DISCUSSION

### 3.1 Th2 predominant cytokines expression in pregnancy

Sex steroids are pleiotropic hormones influencing the development, maturation, activation and death of immune cells. Recent studies indicate that one of the mechanism

involve the modulation of the secretion of immunoregulatory cytokines by hormone. The present study using flow cytometry showed increased numbers of Th2 and TGF $\beta$  expression from peripheral blood and spleen in the third trimester of pregnancy. They are in line with many reports on human pregnancy, indicating a Th2 predominance in pregnancy. Cytokines are not only restricted to T cells, other MNCs can also express a variety of cytokines. So our results gave a general repertoire of cytokine expression in MNCs instead of only T cells in previous reports.

The increased production of Th2 cytokines plays an important role in the establishment of tolerance in pregnancy. IL-10 induces the downregulation of MHC II and the decrease in the expression of ICAM-1, CD80 and CD86 on antigen-presenting cells (APC). Another important Th2 cytokine, IL-4, can inhibit the development of type 1 cells, further promotes a “tolerant” type 2 phenotype, which fosters the development of anergy rather than activation. TGF $\beta$ -expressing cells have repeatedly been shown to perform protective and regulatory effects in autoimmunity. So the superior IL-10 and TGF $\beta$  production in pregnant rats may contribute to the tolerance in pregnancy.

So far, an agreement has been achieved among the findings in peripheral blood Th1 cells in normal pregnancy. It is reported that no difference in the level of IFN $\gamma$ -producing cells throughout the overall gestation period<sup>[5]</sup>, in contrast, a reduced numbers of IFN $\gamma$ -positive cells was found in the third trimester in women by stimulating the periphery MNCs with 12-myristate-13-acetate (PMA)<sup>[6]</sup>. In our case, the number of IFN $\gamma$  positive cells did not change in pregnancy. Our results reflected the true features of IFN $\gamma$  expression *in vivo* by detecting the freshly prepared MNCs with flow cytometry and excluded any interference of antigen stimulus.

### 3.2 Changes in surface markers on MNCs

The antigen-presenting ability is crucial for the generation of an immune response and depends on MHC II expression on the surface of monocytes and other antigen-presenting cells. Monocytes, fibroblasts, endothelial cells, T cells, astrocytes and microglial cells can be induced to express MHC II by a variety of stimuli<sup>[7]</sup>. Significantly downregulated MHC II in spleen MNCs was revealed in pregnancy. The relative absence of specific antigen presentation could prevent T-cell mediated rejection response. The immune response in pregnancy is thus in the form of costimulatory surface molecules or cytokines in the absence of MHC II.

The integrin chain CD11c is a marker for most den-

dratic cells (DC). DC subsets may provide T cells with the different cytokine/molecule microenvironments that determine the classes of immune response. In humans, monocyte-derived CD11c<sup>+</sup> DC polarizes naive T cells predominantly towards a Th1 profile, whereas the CD11c<sup>-</sup> DC subset induces T cells to predominantly produce Th2 cytokines<sup>[8]</sup>. Thus the decrease of CD11c<sup>+</sup> cell in periphery MNCs in our study may be partly responsible for Th2 cytokines shift in pregnancy.

### 3.3 Ag specific proliferation of MNCs from blood, spleen and lymph nodes

MNCs from virgin rats showed an increasing tendency of proliferation in presence of autoantigens compared to those cultured in pure medium, but never reach statistical significance. The same thing went to the MNCs from pregnant rats. The absence of antigen specific proliferation of MNCs from blood and spleen in our study may be due to the lack of memory T cells in virgin rats. Both the spontaneous and antigen specific proliferation in pregnant rats were undiminished, arguing against an overall immune deficit in pregnancy.

To our surprise, the mesenteric lymph nodes MNCs in pregnant rats obtained higher proliferative ability. We supposed that the innate immune system is activated in pregnancy. Increasing evidences show that pregnant animals, far from being immunosuppressed, the maternal innate immune system are activated systemically. For example, there are increased numbers of monocytes and granulocytes from the first trimester onwards<sup>[9]</sup>. So the higher proliferation of lymph nodes MNCs in pregnant rats can help protect the mother against infection<sup>[10]</sup>.

### 3.4 Decreased anti-AChR and anti-OVA antibody in pregnant rats

B lymphopoiesis is remarkably downregulated during pregnancy and all precursor populations beyond the early pro-B cell stage are affected<sup>[11]</sup>. Sustained exposure to estrogen caused a reduction of IL-7 responsive cells and pre-B cells in the marrow, as well as newly made B cells in the periphery<sup>[12]</sup>. In our study, the suppression of antibody production may account for the protection of MS by estrogen. It is surprising that MBP-specific antibody-secreting cells couldn't be detected in spleen using MBP protein. Considering that pregnancy is a special tolerant situation, the antibodies production may be not so simple as the effect of ex-treated sex hormones. The reason deserves further investigation.

In general, pregnancy is a special process character-

ized by some immunological variations, which may contribute to the remission of Th1-mediated autoimmune diseases as MS in pregnancy.

### Acknowledgment:

We thank the department of immunology of our university for providing the facility.

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