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Expression of indoleamine 2,3-dioxygenase in pregnant mice correlates with $CD4^+CD25^+Foxp3^+$ T regulatory cells

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Abstract. – OBJECTIVE: Indoleamine 2,3-dioxygenase (IDO) initiated tryptophan degradation in the placenta has a role in the prevention of allogeneic fetus rejection by T-cells. The present study aimed to investigate the relationship between IDO and $CD4^+CD25^+Foxp3^+$ T cells in pregnant mice.

MATERIALS AND METHODS: The percentage of $CD4^+CD25^+Foxp3^+$ T cells in peripheral blood mononuclear cells (PBMC) and IDO mRNA levels were detected in pregnant mice. The non-pregnant mice were used as control in this study. To confirm the effect of IDO, 1-methyl-trytophan (IDO inhibitor) was used in this study.

RESULTS: The percentage of $CD4^+CD25^+Foxp3^+$T cells in PBMC in pregnant mice was significantly higher than this in non-pregnant mice controls from day-6 to the end of the study ($p<0.05$). IDO mRNA levels in PBMC also markedly increased after pregnancy. The upregulation of IDO expression reached a maximum at day 18 after pregnancy ($p<0.05$). Compared to the pregnant group, the inhibitor could significantly decrease the IDO expression and Treg percentage ($p<0.05$). There was a positive association between IDO mRNA and $CD4^+CD25^{+-}$ $Foxp3^+$ T cells percentage.

CONCLUSIONS: The results suggested IDO might play a role in the generation of $CD4^+CD25^{+-}$ $Foxp3^+$ T cells in pregnant mice.

Key Words
IDO, Foxp3, Fetus rejection.

Introduction

The enzyme indoleamine 2,3-dioxygenase (IDO) is a monomeric hemoprotein with a molecular mass of about 45 kDa. IDO is involved in the kynurenine metabolism pathway, which catalyzes the first and rate-limiting step of the essential amino acid L-tryptophan, the oxidization of the pyrrole moiety of tryptophan to N-formylkynurenine[1,2]. IDO is widely expression in many tissues under various pathological conditions associated with immune activation, and it has been demonstrated that induced IDO can inhibit proliferation of many pathogens, including tumors and intracellular parasites, by depletion of tryptophan[2-4]. IDO is higher expressed in the lung, the intestine, and the placenta[5-7]. Various populations were in poor health, suffering from IDO[8,9]. Many studies[10-12] demonstrated that IDO contributes to maternal tolerance in pregnancy. However, the role of IDO has not been fully elucidated, especially in pregnancy process. Previous studies showed that, on the one hand, IDO generates metabolic products that induce regulatory T (Treg) cells, on the other hand, Treg cells can induce IDO expression in placenta. This suggests the presence of a positive feedback loop and raises the question of the limitation of this mutual interaction[13]. The most cited publication on IDO study was Munn et al[14] who proposed that the placental IDO might play an important role in the prevention of allogeneic fetus rejection by T-cells. Also kynurenine, the product of tryptophan, displays immunosuppressive properties by generating Treg cells[15,16]. Naturally occurring Treg cells, which characteristically express the nuclear transcription factor forkhead box protein 3 (Foxp3) and express CD4 and CD25 on the cell-surface[17]. Treg cells can secrete pro-inflammatory cytokines, including interlukin-10 (IL) and transforming growth factor-β (TGF-β) to suppress the allergen-induced specific T cells activation, and also suppress the effector cells of allergic inflammation, such as basophils, and eosinophils[18-20]. Thus, in the present study, we aimed to investigate the interactions between IDO and Treg cells in feto-maternal tolerance. Also, the mRNA level of IDO and $CD4^+CD25^{+-}$ $Foxp3^+$Treg cells in peripheral blood mononuclear cells (PBMC) were measured in this study.

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Materials and Methods

Mice

Eight-week-old CBA/J female and BALB/c male mice were purchased from Institute of Laboratory Animal Science (ILAS), Chinese Academy of Medical Sciences (CAMS). The mice were maintained in an animal facility with controlled humidity, light (12/12 h light/dark) and temperature. Animals were fed mouse chow and tap water *ad libitum*. The present study was conducted with approval from the Animal Ethics Committee of the University. 60 female CBA/J mice were bred with 30 BALB/c male mice in one cage, and the vaginal plugs in individual mated female mice were examined daily to determine pregnancy. The day of visualization of a plug was designated as day 0 of pregnancy. The inhibitor group was intraperitoneally (i.p.) injected with 1-methyl-tryptophan (1-MT) 3.75 mM after pregnancy.

Blood sampling and separation of peripheral blood mononuclear cells (PBMC)

The pregnant mice were killed by spinal dislocation, and peripheral blood was collected into heparin anticoagulant tubes. PBMC were isolated from whole blood by density gradient centrifugation.

Reverse transcriptase PCR analysis of IDO mRNA

Total RNA was isolated from peripheral blood mononuclear cells (PBMC) with Trizol reagent (Gibco, Grand Island, NY, USA), and 1 μg of each isolated RNA was subjected to cDNA synthesis. RT cDNA synthesis was conducted in a 14 μl reaction buffer, containing 1 μl reverse transcriptase (50 U) and 1 μl oligo (dT) primer, according to manufacturer's instructions (TaKaRa, Otsu, Shiga, Japan). With the obtained for cDNA as a template, the relative expression levels of IDO from PBMC were determined by PCR. The sequence of the primers for RT-PCR are as follows, IDO, Forward, 5'-GCGCTGTTGGAAATAGCTTC-3', and Reverse, 5'-CAGGACGTCAAAGCACTGAA-3'; β-actin, Forward, 5'-AGAGCTACGAGCTGCCTGAC-3'and Reverse, 5'-AGTACTTGCGCTCAGGAGGA-3'. Each 20 μl reaction system comprised 2 μl of cDNA, 10 μl SYBR Premix Ex Taq II, 10 μmol/l of both sense and antisense primers. Amplification parameters of IDO were as follows: 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 51°C for 30 s, 72°C for 30 s, and finally 72°C for 5 min. Amplification parameters of β-actin were as follows: 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 30 s, and finally 72°C for 5 min. For normalization, β-actin was used to normalize mRNA. The final PCR products were analyzed on an agarose gel, and the relative intensity was determined using semiquantitative densitometry. Results were calculated using the $2^{-\Delta\Delta Ct}$ method.

Treg cells analysis by flow cytometry

For analysis of Treg cells, about 10^6 freshly isolated PBMC cells were first stained with fluorescein isothiocyanate (FITC)-labelled CD4 and phycoerythrin (PE)-labelled CD25 antibodies, incubated at 4°C for 20 min in the dark, then washed in cold flow cytometry staining buffer. For further Foxp3 staining, cells were firstly fixed and permeabilized by a commercial cell fixation/permeabilization kit at 4°C for 30 min in the dark using PE-Cy5-labelled Foxp3 antibody. Otypic controls (Rat IgG2aPE-Cy5-labelled) were used as negative controls. The stained cells were analyzed using flow cytometry. All flow cytometry antibodies and reagents were from eBioscience Inc. (San Diego, CA, USA) and flow cytometric analysis was performed on a FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using Cellquest Pro software.

Statistical Analysis

Data are expressed as mean ± SD. Statistical differences were evaluated by software SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using one-way analysis of variance, and $p<0.05$ were considered as statistically significant. The LSD test was used as a post-hoc test to confirm ANOVA.

Results

Reverse transcriptase PCR analysis of IDO mRNA

The mRNA expression levels of PBMC were assessed by RT-PCR. There was no difference from day 0 to the end of IDO mRNA expression levels in the PBMC in control group ($p>0.05$, Figure 1). Compared with the day 0 in pregnancy group, the IDO mRNA expression levels were found to rise during the pregnancy process, and this difference was significant ($p<0.05$, Figure 1). The upregulation of IDO expression reached a

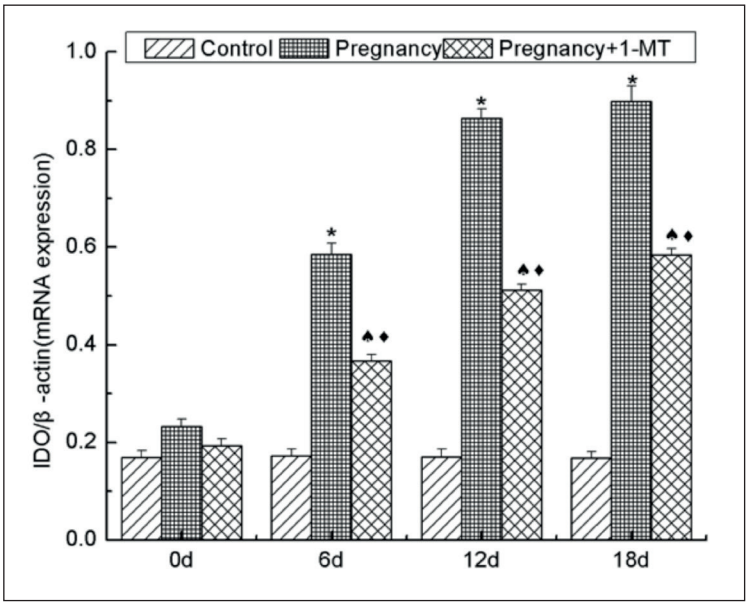

Figure 1. RT-PCR analysis of IDO mRNA expression during the gestation period (n=6), *p<0.05, compared with pregnancy group (day 0); ♠p<0.05, compared with pregnancy+1-MT group (day 0); ♦p<0.05, compared with pregnancy group (at same day after pregnancy).

maximum at day 18 after pregnancy (p<0.05, Figure 1). Meanwhile, the inhibitor 1-MT treatment could remarkably reverse the trend of IDO mRNA expression levels after pregnancy (p<0.05, Figure 1). These results demonstrate that the pregnancy in mice could increase the IDO mRNA levels in PBMC.

Treg cells analysis by flow cytometry

To investigate the potential role of Treg cells during pregnancy, we first examined the number of Treg lymphocytes in PBMC, seen as Figures 2 and 3. For $CD4^+CD25^+Foxp3^+$ Treg cells, the numbers were very low at day 0 till to the end of the experiment in control group, and also there were no significant changes (p>0.05, Figure 3). In the pregnancy group, there was remarkably increased the number of Treg during the pregnancy when compared to day 0 (p<0.05, Figure 3). When pregnant mice treated with 1-MT, Treg cells were significantly reduced from day 6 to day 18 (p<0.05, Figure 3). However, the mean number of Treg cells was increased in the pregnancy+1-MT group compared to control group from day-6 to the end of the study (Figure 3). These results again suggest that pregnancy could increase the Treg cells in PBMC, while the inhibitor could reverse this trend in our work.

Correlation between Treg cells and IDO mRNA expression levels

The Treg cells number and IDO mRNA levels both increased during the gestation period. As showed in Figure 4, there was a strong positive correlation between Treg cells number and IDO mRNA expression in pregnancy group and inhibitor group (R^2=0.98669, 0.98404, respectively, Figure 3), while there was no correlation in control group.

Discussion

This study demonstrated the detailed time course of IDO expression during the development of embryo/fetus. Our results showed that IDO mRNA expression is significantly increased from 6-day post-coitus (3 dpc) to 18 dpc compared to no pregnant mice. Furthermore, the level of Treg cells was consistent with that of IDO, indicating a relationship between IDO expression and Treg cells in pregnant mice. Our data revealed that IDO and Treg cells were markedly downregulated in the 1-MT group compared to pregnant mice group. The function of IDO in pregnancy has been described as a regulator in maternal tolerance, control of allograft rejection[2-6]. The majority of people in renal disease was checked with IDO[21-23]. In both humans and mice, IDO is generated by dendritic cells (DCs). In the early pregnancy, the IDO expression is restricted exclusively to immediately sub trophoblastic capillaries, and it increases with advancing gestational age[18]. Also, the increase in protein expression correlates with the placental kynurenine-to-Trp ratio, a surrogate measure of IDO activity. In

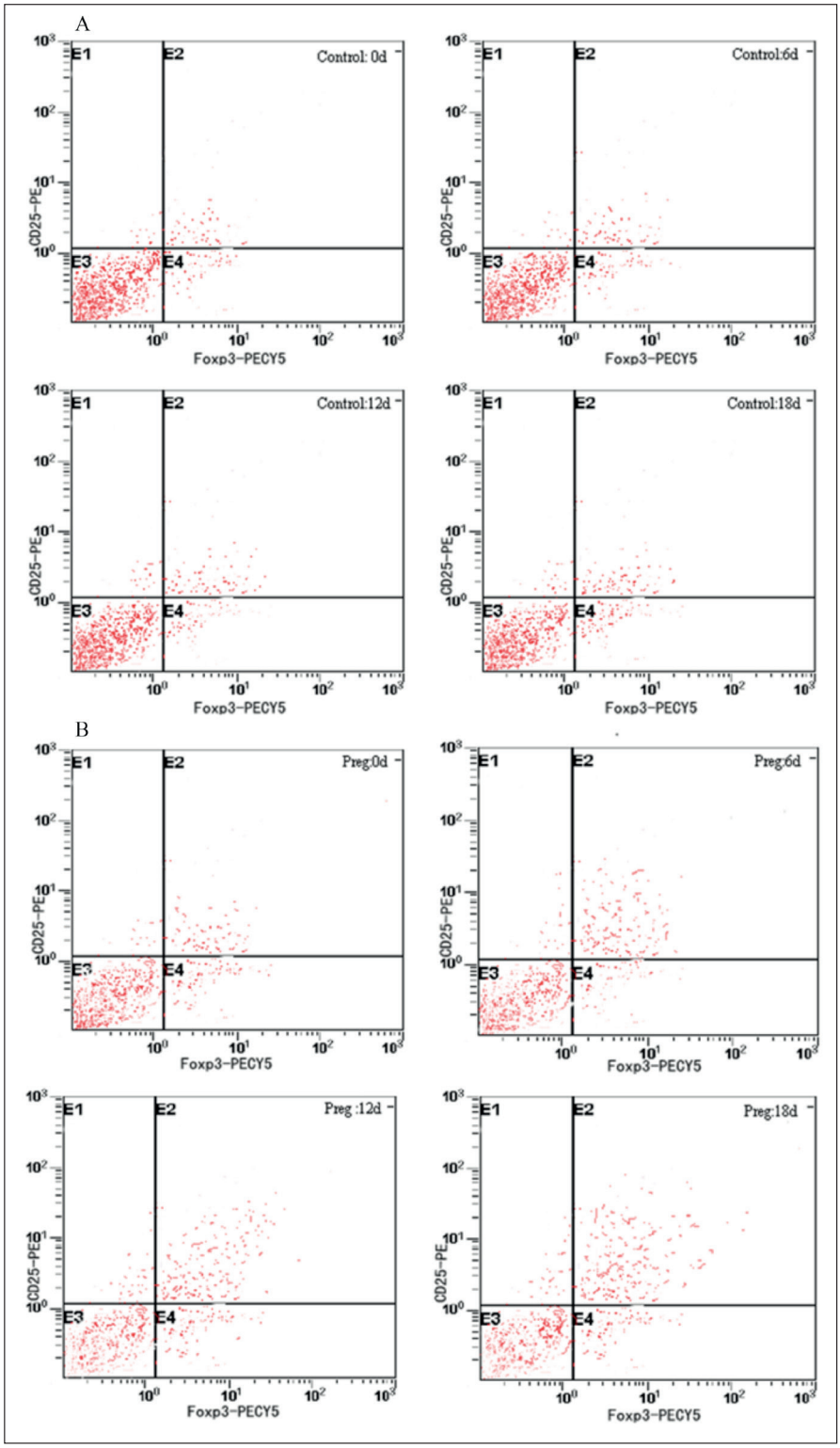

Figure 2. Flow diagram of CD4 + CD25 + Foxp3 + Treg cells in all groups of mice peripheral blood. **A**, Control group; **B**, Preg group; **C**, 1-MT group.

agreement with these studies, the data presented here showed that the IDO mRNA expression in PBMCs increased during the gestational period compared to non-pregnant mice. During the development of fetus placenta, IDO was significantly increased. This could be sufficient to induce immune tolerance in pregnancy. In the present report, injection with the IDO inhibitor, 1-methyl-trptophan, led to a decrease in IDO expression of pregnant mice, which further demonstrated the importance of IDO to maternal-fetal. Besides the up-regulation of IDO during the pregnancy,

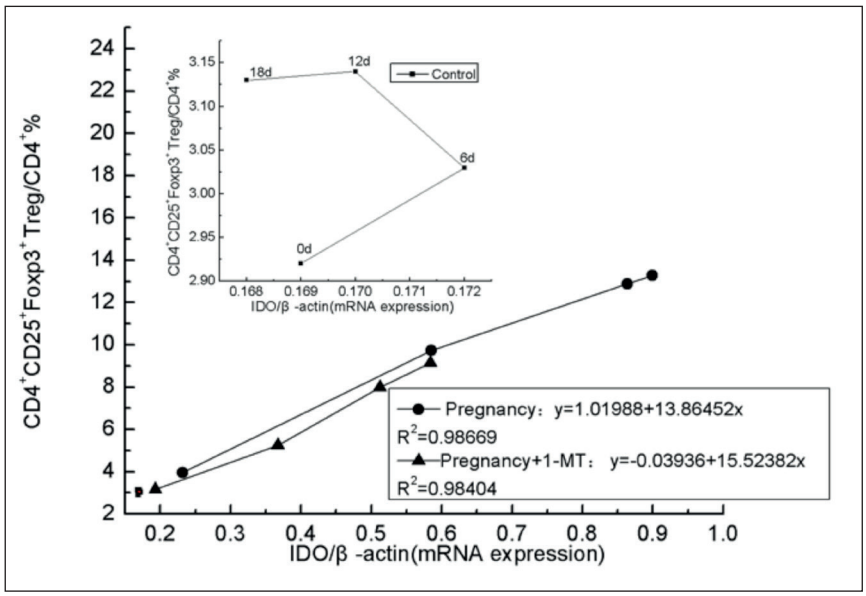

Figure 4. The correlation between IDO and $CD4^+CD25^+Foxp3^+$Treg cells in PBMC. The illustration is the group of health and not pregnant mice.

leading to differentiation to $CD4^+CD25^+Foxp3^+$T cells. The $CD4^+CD25^+$ subpopulation of T cells has been shown to be crucial in self-tolerance and also to prevent allograft rejection[23-25]. Two theories on the function of IDO in Treg generation have been proposed. One is that IDO leads to tryptophan depletion, and this relative starvation leads to cell cycle arrest in some populations, favoring the generation of Tregs[26]. The second one is that tryptophan catabolites themselves have a more direct role in the generation of Tregs[27,28]. These are not mutually exclusive possibilities, and each might have a role in the maternal tolerance in pregnancy. It is still plausible that the tryptophan depletion continues to play a role in the IDO-dependent generation of $CD4^+CD25^+Foxp3^+$ T cells. Also in this study, a positive correlation is found between the IDO expression and the generation $CD4^+CD25^+Foxp3^+$ T cells. The combination of these IDO enzymes and $CD4^+CD25^+Foxp3^+$ T cells have important consequences for the establishment and maintenance of feto-maternal immune tolerance.

Conclusions

The results presented here indicate that the IDO enzyme and $CD4^+CD25^+Foxp3^+$ T cell play an important role in feto-maternal immune tolerance. This transient expression of IDO should be sufficient to induce immune tolerance. Studies on the relationship of IDO and $CD4^+CD25^+Foxp3^+$ T cell will deepen our understanding of tryptophan catabolism in mother-fetus interaction.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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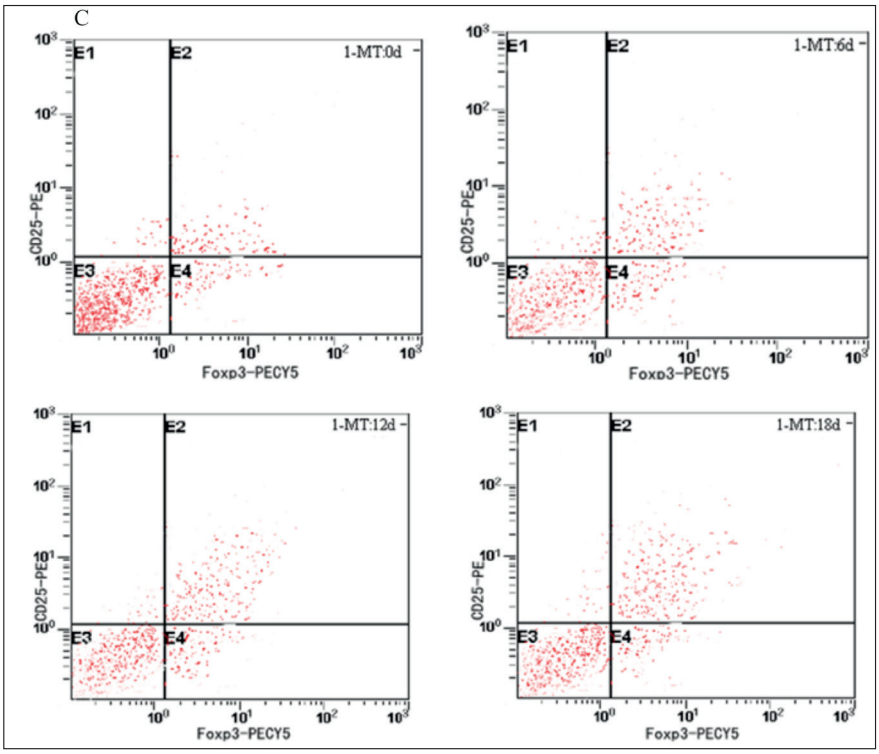
Figure 2 (Continued).



the increases of $CD4^+CD25^+Foxp3^+$ T cells were found in the present work. This indicated that $CD4^+CD25^+Foxp3^+$ T cells could be a factor in the maternal-fetal tolerance. When the pregnant mice treated with the inhibitor of IDO, the number of $CD4^+CD25^+Foxp3^+$ T cells was significantly reduced in our study. Therefore, we suggested that the up-regulation of IDO and the increase of the number of $CD4^+CD25^+Foxp3^+$ T cells have a relationship. The results demonstrated that IDO expression was positively related to the number of $CD4^+CD25^+Foxp3^+$ T cells (Figure 4). The results were consistent with Mezrich et al[24] who demonstrated how IDO, via the kynurenine pathway, leads to Treg generation. Kynurenine binds to the aryl hydrocarbon receptor (AHR) in T cells,

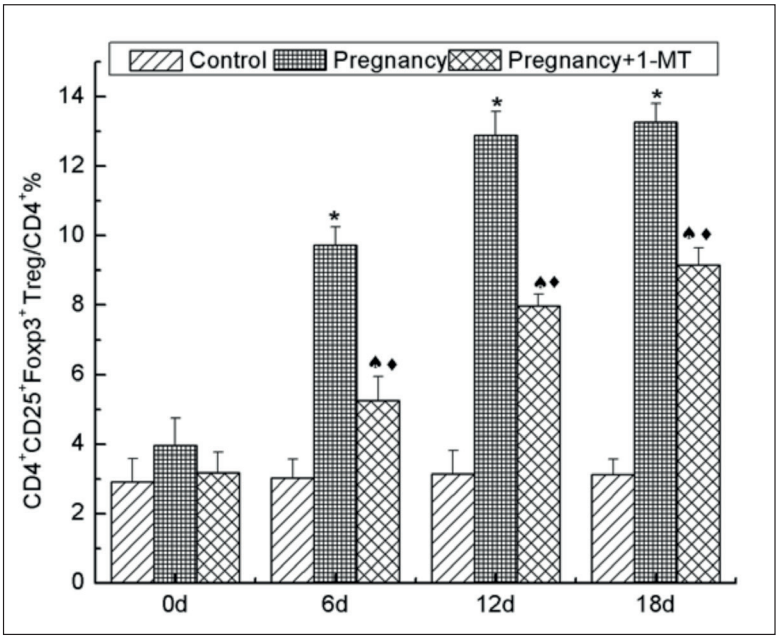


Figure 3. The percentage of $CD4^+CD25^+Foxp3^+$ Treg in PBMC of mice (n=6), $*p<0.05$, compared with pregnancy group (day 0); ♠$p<0.05$, compared with pregnancy+1-MT group (day 0); ♦$p<0.05$, compared with pregnancy group (at same day after pregnancy).

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