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The regular distribution and expression pattern of immunosuppressive cytokine IL-35 in mouse uterus during early pregnancy

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Abstract

Cytokines within the uterus are critical in the maternal-fetal immune regulation. Immunosuppressive cytokine IL-35 was recently discovered inhibitory cytokine, which were pivotal in the establishment of immune tolerance against self-antigens and antigens encountered in foreign implantation. In order to analyze the role of IL-35 in maternal-fetal immune tolerance, the expression patterns of IL-35 in mouse endometrium were studied during early pregnancy by immunohistochemistry, ELISA and quantitative real-time PCR. As results, we found that IL-35 positive cells in the uterus showed significant distribution difference after fetal implantation, which mainly distributed in luminal epithelium and glandular epithelium of mouse uterus from gestational day 1 to 2, and glandular epithelium and stroma from gestational day 4 to 7. The number of positive cells, immunoreactive scores, protein and mRNA expression of IL-35 showed firstly increased and then decreased with the increase of pregnancy day. The largest contents of IL-35 in the uterus were detected on gestational day 4. Compared with non-pregnant mice, pregnant mice showed the significantly increased mRNA expression of Ebi3 (Epstein–Barr virus-induced gene 3, IL-35 subunit) in the endometrium on gestational day 2 and the highest level of expression on gestational day 4. The mRNA expression of p35 (IL-35 subunit) was significantly lower than that of Ebi3 gene and showed the inconsistent change from gestational day 5 to 7. However, the significant correlation existed between the immunohistochemical expression, contents and mRNA expression of IL-35. These results indicated that IL-35 contributed to the establishment and maintenance of maternal-fetal tolerance during early pregnancy.

Keywords: IL-35, uterus, distribution, expression, early pregnancy.

Introduction

Embryo implantation in placental mammals is the interaction process among active embryo, receptive uterus, and endometrium. In this process, successful implantation depends upon the developmentally and physiologically coordinated interaction of two distinct yet physically opposed organ systems (the placenta and the uterus) and the successful establishment and maintenance of maternal-fetal immune tolerance [1]. The establishment of maternal-fetal immune tolerance refers to the process that the uterine immune system produces temporary immunosuppression, prevents immune-mediated rejection of the semiallogeneic fetuses, and protects fetus from being attacked by maternal immune cells [2, 3]. Although the complex mechanism of maternal-fetal immune tolerance has not been clearly elucidated yet, it is believed that the T-cells and many cytokines in the maternal-fetal interface play an important role in maternal-fetal immune tolerance [4].

Regulatory T-cells (Treg cells) are a unique subset of CD4+ T-cells that are essential for inhibited immune response by influencing the activity of other cell types [5] and by using secreted cytokines [6, 7]. According to the different types of secretion of cytokines, Treg cells are classified into naturally occurring CD4+CD25+ Treg cells, interleukin 10 (IL-10) secreting Treg cells and transforming growth factor β (TGF-β) secreting Treg cells. Current studies have found that the CD4+CD25+ Treg cells played a pivotal role in the immune tolerance [8]. The CD4+CD25+ Treg cells can: convert non-Treg cells into suppressive cells; inhibit proliferation and activation of conventional T-cell (Tconv) independent of CTLA-4 [9]; regulate of autoimmune and inflammatory diseases by secretion of immunosuppressive cytokines TGF-β and IL-10 [10, 11]; maintain the self-tolerance by suppressed the activity and expression of potentially pathogenic self-reactive T-cells [12]. The CD4+CD25+ Treg cells not only participated in preventing autoimmune diseases and limiting chronic inflammatory diseases, such as type 1 diabetes and inflammatory bowel disease (IBD) [13], but also suppressed beneficial responses by preventing sterilizing immunity to certain pathogens and limiting antitumor immunity [14, 15]. The CD4+CD25+ Treg cells express the lineage-specific transcription factor Foxp3, which is required for their development and function [16]. Foxp3 can convert naïve T-cells to the Treg cell lineage [17]. When Foxp3 deletion or mutation, lead to the lack of CD4+CD25+ Treg cells, which further induce the same autoimmune syndrome [18, 19]. More important, some studies have confirmed that the CD4+CD25+ Treg cells contribute to the establishment of maternal-fetal immune tolerance by suppressing rejective reaction of maternal to fetal allograft [20]. As the pregnancy continued, the CD4+CD25+ Treg cells were increase in the peripheral blood and the uterine deciduas [21, 22]. The lack or significant decrease of CD4+CD25+ Treg cells can broke the maternal-fetal immune tolerance, lead to early failure of gestation and abortion [23, 24].

IL-35 (Interleukin-35) is a recently discovered inhibitory cytokine produced by CD4+CD25+Foxp3+ Treg cells and contributes to their suppressive function [25]. IL-35, as a member of the IL-12 (Interleukin-12) cytokine family, is a heterodimeric protein composed of Ebi3 (Epstein–Barr virus-induced gene 3) and IL-12p35 subunits, which are encoded by two separate genes, ebi3 and p35, respectively [26]. But, IL-35 is different from other family members for it is produced in non-stimulated Treg cell and it is the immunosuppressing cytokine in mice [27]. IL-35 can suppress the proliferation and activities of Th1 and Th17 cells, promote the expansion of Treg cells by increasing the expression of anti-inflammatory cytokines IL-10 and TGF-β, and convert naïve T cells into IL-35-induced regulatory T-cells (iTr35 cells), thus contributing to the establishment of immune tolerance [28–30]. However, more significantly, we found that IL-35 was constitutively expressed in human trophoblasts and that Ebi3 and p35 mRNAs were co-expressed in trophoblast cells during pregnancy [31]. Placental trophoblast cell are the only cell of fetal origin in direct contact with maternal immune system and the most important cell in early pregnancy. To further investigate the dynamic expression and distribution patterns of IL-35 in the endometrium and the potential role in the establishment and maintenance of maternal-fetal immune tolerance during early pregnancy, we detected IL-35 expression and distribution in the 7-day mouse uterus by immunohistochemical staining, ELISA and quantitative real-time PCR in this study.

Materials and Methods

Animals

The research was conducted at the Anhui Science and Technology University, Anhui, China during the autumn (September and October) of 2013 and lasted for 30 days.

A total of 120 Kunming mice (included 80 females, 22±2 g body weight (BW); 40 males, 25±2 g BW; eight weeks old) were obtained from the Laboratory of Animal Research Centre of Nanjing Qinglongshan and housed in SPF Laboratory Animal House at the Anhui Science and Technology University. These mice were mated with the same male variety (females/males 2:1) to produce pregnancy after one-week adaptive feeding. The mice were divided into eight groups according to the days of pregnancy, namely, non-pregnancy (P0, n=10) and gestational day 1–7 (P1–P7, n=10), and maintained under standard conditions. The light/dark cycle was 14/10 hours (lighting time: 06:30–20:30). The vaginal tied was observed in the vagina at 07:00 on the next morning after mating, the mating day was defined as pregnant day 1. The mice had free access to distilled water and "Tangshan Longquan©" experimental mice feed (Nanjing Qinglongshan Animal Breeding Farm, Nanjing, GB14924.1-2001). The feed composition was provided as follows: moisture ≤10%, crude protein ≥18.15%, crude fat ≥4.03%, crude fiber ≥5.12%, and crude ash ≥7.94%.

All experimental procedures were conducted according to the *Guidelines for The Care and Use of Laboratory Animals* in Anhui Laboratory Animal Management Center, China, and conformed to the *National Institutes of Health Guide for Care and Use of Laboratory Animals*. Each experimental protocol was statistically designed to use the minimal number of animals.

Sample collection

The blood samples were collected and the uterus was carefully removed after all the animals were anesthetized. Blood (1 mL) from each mouse was collected in the Eppendorf tube from orbital venous, clotted at room temperature (25^0C) for 60 minutes, and centrifuged (Beckman Allegra X-30R; Beckman Coulter Trading (China) Co., Ltd.) at 1200×g for 15 minutes at 4^0C. The part of uterus samples were fixed in 4% paraformaldehyde phosphate buffer (0.1 M, pH 7.4) at room temperature (25^0C) for immunohistochemical staining, and the endometrium of other part was peeled and frozen in liquid nitrogen for ELISA and quantitative real-time PCR.

Immunohistochemical staining

IL-35 expression and distribution were detected with Streptavidin–Biotin-peroxidase complex method. After 4% paraformaldehyde phosphate buffer fixation, the uterus samples were dehydrated by graded ethanol treatment, cleared in xylene, paraffin embedded. Serial paraffin cross-sections were made with a thickness of 6-μm and placed on unilateral frosted microscope slides. The sections were then deparaffinized in xylene, rehydrated in a series of ethanol solutions, and subjected to antigen retrieval through 15-minute boiling in target retrieval solution (citrate buffer, pH 6) in a microwave oven. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol for 30 minutes. Nonspecific binding was blocked by incubation with 10% normal goat serum in phosphate buffered saline (PBS, pH 7.4) for 30 minutes. The sections were incubated overnight at 4^0C with rabbit anti-mouse IL-35 polyclonal antibody (pAb) (USCN, Wuhan, China) according to the dilution ratio of 1:100, washed in PBS, and then incubated in biotinylated secondary antibody (CWBIO, Beijing, China). Streptavidin-peroxidase (CWBIO, Beijing, China) was bound to the Biotin of the secondary antibody, and the peroxidase reaction was developed with DAB (Sigma, USA) and nuclei were counterstained with Hematoxylin. In the controls, the primary antibody was replaced by rabbit isotype-matched irrelevant IgG and used as negative controls. Samples were observed with Olympus BX51 microscope and images were obtained with DP 73 digital camera (Olympus Sales & Service Co., Ltd. Beijing, China).

We assessed results of the immunostaining by used the number of immunoreactive cells and the immunoreactive score (IRS) of the Remmele and Stegner [32]. For counting of immunoreactive cells, the amount of all positive cells per unit area was counted through observing the 25 random fields from five cross-sections of the uterus of each mouse in each group. According to immunoreactive score, the quantitative evaluation of IL-35 was performed following a score based on two criteria: the reaction intensity of positive cells (0 for negative, 1 for weakly positive, 2 for moderately positive, 3 for strongly positive) and the percentage of the positive staining areas in relation to the whole areas in some fields (0 for 1–10% reactivity, 1 for 10–25% reactivity, 2 for 25–40% reactivity, 3 for 40–75%, 4 for 75–100% reactivity). The

final immunoreactive score was determined by multiplying the positive intensity and the positive area extent scores, yielding a range from 0 to 12. The quantification of the immunoreactivity of luminal epithelium, glandular epithelium and stroma were performed by evaluating percentages of the IL-35-positive cells in all positive of the endometrium.

ELISA assay

The endometrium tissue was homogenized and the supernatant was separated by centrifugation at low temperature. The tissue supernatant and the serum were stored at -80^{0}C for cytokine assay. IL-35 concentration was measured in duplicates with commercial ELISA kits (R&D, USA) according to the manufacturers' instructions and calculated with standard protein values in pg/mL. The final results were statistically analyzed with one-way ANOVA. The correlation coefficient of the standard curve was 0.9909 (Figure 1) and the intra-coefficients of variation were 9.36%. The concentrations of IL-35 were represented by tissue supernatant in pg/mL.

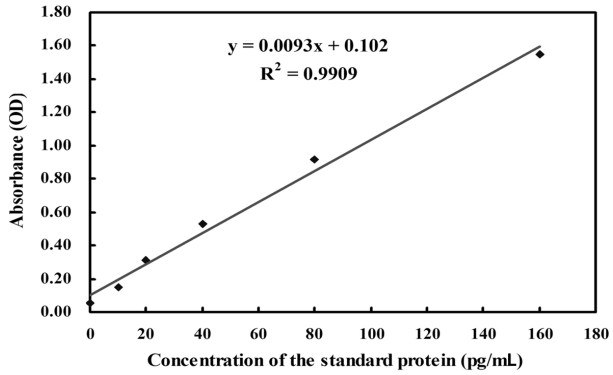

Figure 1 – ***The standard curve of ELISA assay for detected IL-35 concentration. The correlation coefficient (R^2) of the standard curve was 0.9909.***

Quantitative real-time PCR

Total RNA was extracted from frozen endometrium tissue with TRIzol Reagent (Invitrogen, USA). The first-strand cDNA was synthesized by Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo fisher, USA) in a volume of 20 μL. The cDNA was used in quantitative real-time PCR (qPCR) reaction to assess the expression of Ebi3 and p35 subunits of IL-35 in uterine tissues. The used primers were provided as follows: forward primer (5'-GCT CCC AAC TCC ACC AGA T-3') and reverse primer (5'-CGG CTT GAT GAT TCG CTC-3') for Ebi3 (NM_015766), which produced the 231-bp product; forward primer (5'-AGT TTG GCC AGG GTC ATT CC-3') and reverse primer (5'-TCT CTG GCC GTC TTC ACC AT-3') for p35 (NM_ 001159424), which produced the 101-bp product. The mouse GAPDH was used as internal control from parallel samples and its sequences were obtained from *Primer Bank* of *The Massachusetts General Hospital* (http:// pga.mgh.harvard.edu/primerbank/index.html). The primer sequences were as follows: GAPDH forward primer (5'-CAT GTT CCA GTA TGA CTC CAC TC-3') and GAPDH reverse primer (5'-GGC CTC ACC CCA TTT GAT GT-3'), which produce a 136-bp product (*Primer Bank ID*: 6679937a2). The cDNA samples were subjected to 40 cycles of amplification in an ABI 7500 Sequence Detection System instrument. The SYBR Premix Ex TaqTM Kit (Taraka, China) were used according to the protocol provided by the manu-facturer. Melting curve analysis was used to confirm amplification specificity. The relative mRNA expression was determined by the comparative cycle threshold (CT) method. The quantification data were analyzed with ABI prism SDS analysis software 2.0 (Applied Biosystems, USA) and the expression of related target genes was normalized through the expression of GAPDH (NM_008084.2) and determined by the following formula: $2^{-\Delta\Delta CT}$.

Statistical analysis

All experimental data were analyzed using SPSS version 18 software (SPSS Inc., Chicago, IL, USA). The homogeneity of data variances was analyzed using Levene's test, performed in SPSS *via* ANOVA using GLM (general linear model) procedure and normality of data distribution was tested by the Kolmogorov–Smirnov test. The statistical significance in this study was compared by the Dunnett's *t*-test. The differences between non-pregnant and gestational day 1–7 were confirmed with the level of significance set at $p<0.05$ and the level of extreme significance set at $p<0.01$. The correlation analysis between detection results of different items were performed by Pearson's test. The level of significant correlation set at $p<0.01$.

Results

Immunocytochemical staining of IL-35 in the mouse uterus during early pregnancy

The distribution of IL-35 was evaluated by immunohistochemistry (IHC) in the mouse uterus during early pregnancy (Figure 2). The majority of positive expression of IL-35 was detected in the glandular epithelium and the minority was detected in the stromal cell and luminal epithelium of endometrium in the non-pregnant and the pregnant mice on gestational day 1 (Figure 2, A and B). From gestational day 2 to 4, the IL-35-positive cells were rapidly increased in the lamina propria, glandular epithelium, and luminal epithelium of endometrium (Figure 2, C–E), and the positive cells were also observed in the myometrium of endometrium on gestational day 2 and 4 (Figure 2, C and E). In particular, on gestational day 4, the IL-35-positive cells were significantly increased in the primary decidual zone (Figure 2E). From gestational day 5 to 7, the IL-35-positive cells were significantly decreased in the glandular epithelium, stroma, and luminal epithelium of endometrium (Figure 2, F–H), but the same changes were not observed in the secondary decidual zone from gestational day 5 to 6. However, the positive expression of IL-35 was obviously reduced in the glandular epithelium, stromal cells, and secondary decidual zone on gestational day 7 (Figure 2H).

Quantitative analysis of IL-35 immunostaining

We analyzed the number of IL-35-positive cells per unit area in the mouse uterus during early pregnancy (Figure 3).

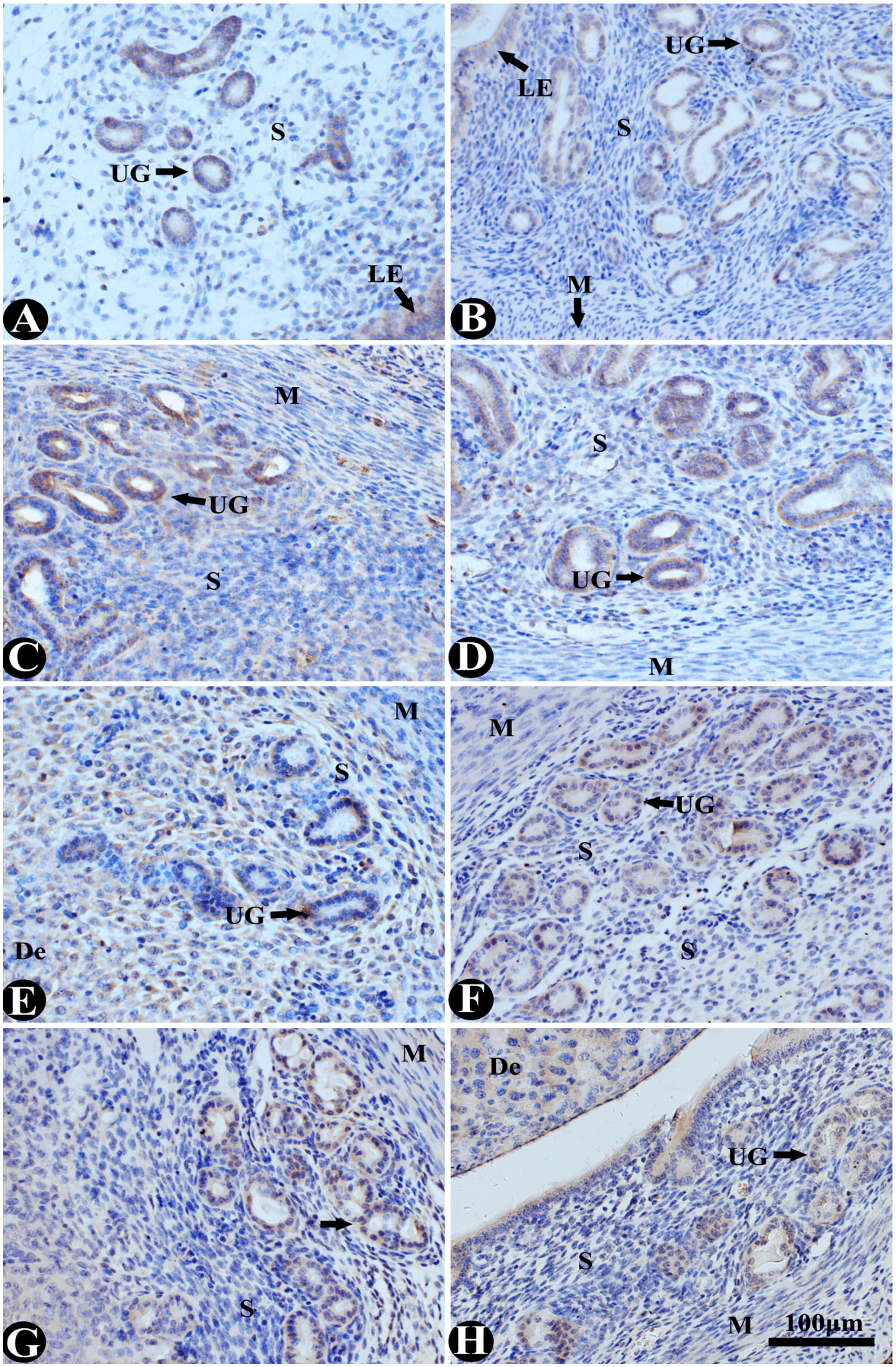

Figure 2 – ***The distribution of IL-35-positive cells in the mouse uterus during early pregnancy. Immunohistochemical staining showed that IL-35-positive cells were significant distribution difference in the uterus from gestational day 1 to 7. (A) Non-pregnant uterus; (B–H) Uterus on gestational days 1 to 7. UG: Uterus gland; S: Stromal; LE: Luminal epithelium; M: Myometrium; De: Decidua. Scale bar = 100 μm.***

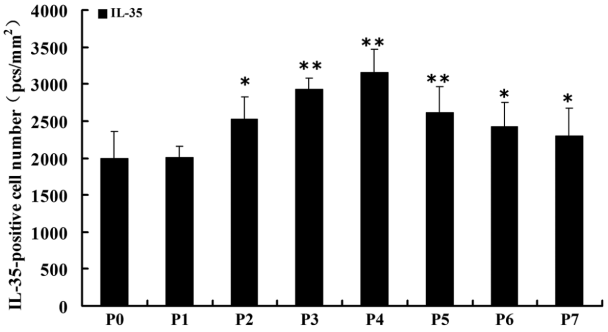

Figure 3 – ***The number of IL-35-positive cells was analyzed in the uterus during early pregnancy. The number of IL-35-positive cell was significantly increased from gestational day 2 to 7 compared to non-pregnant mice (*p<0.021, **p<0.001 respectively), and the number was the largest on gestational day 4 (**p<0.001). P0 represented non-pregnancy; P1–P7 represented gestational day 1 to 7, respectively. **p<0.01, *p<0.05.***

The number of IL-35-positive cells were the least in the uterus of non-pregnant mice, and showed no difference from that of the mouse on gestational day 1 (p=0.943). From gestational day 2 to 4, the quantity of IL-35-positive cells was rapidly increased and 26.83%, 46.59% and 58.55% higher than that of non-pregnant mice (p=0.012, p<0.001 and p<0.001 respectively). The IL-35-positive cells were the highest on gestational day 4. However, the IL-35-positive cells started to decrease from gestational day 5, but the quantity of IL-35-positive cells of the uterus on gestational day 5 to 7 was still 31.23%, 21.38% and 14.95% higher than that of non-pregnant mice (p<0.001, p=0.010 and p=0.020 respectively).

The IRS scores were also used to evaluate the IL-35 positive expression in the endometrium (Figure 4).

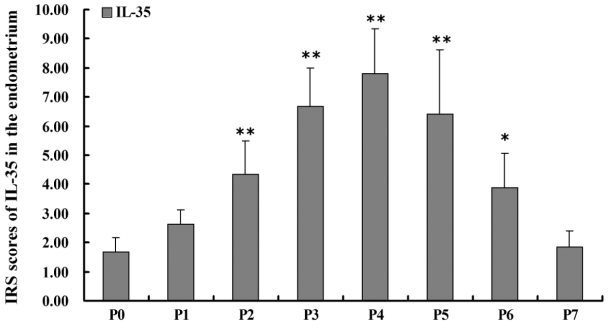

Figure 4 – ***The comparison of IRS scores of IL-35 in the endometrium during early pregnancy. The IRS scores of IL-35 were rapidly increased from gestational day 2 to 4, and decreased from gestational day 5 to 7. P0 represented non-pregnancy; P1–P7 represented gestational day 1 to 7, respectively. **p<0.01, *p<0.05.***

The IL-35 IRS scores were rapidly increased from gestational day 2 to 4, and rapidly decreased from gestational day 5 to 7. However, the IL-35 IRS scores of the endometrium from gestational day 2 to 6 were significantly higher than that of non-pregnant mice (p<0.001 or p=0.03). But, the IL-35 IRS scores of the endometrium from gestational day 1 and 7 were not obviously different with non-pregnant mice (p=0.084, p=0.778).

Further, we evaluated percentages of the IL-35-positive cells of the luminal epithelium, glandular epithelium and stroma in the endometrium (Figure 5).

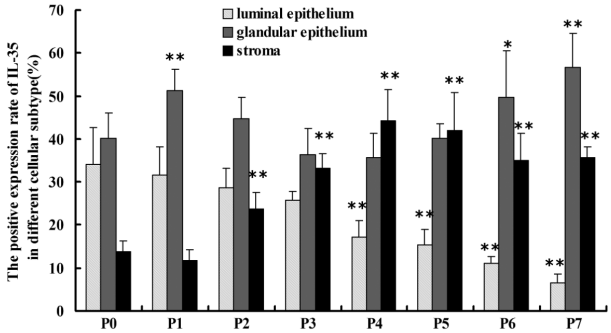

Figure 5 – ***The percentages of the IL-35-positive cells in the luminal epithelium, glandular epithelium and stroma of the endometrium during early pregnancy. The IL-35-positive cells in the luminal epithelium and glandular epithelium were dominant on gestational day 1 to 2. The IL-35-positive cells in the glandular epithelium and stroma were dominant on gestational day 3 to 7. P0 represented non-pregnancy; P1–P7 represented gestational day 1 to 7, respectively. **p<0.01, *p<0.05.***

The percentages of the IL-35-positive cells in the luminal epithelium showed gradually decrease from gestational day 1 to 7, and the percentages from gestational day 4 to 7 were significant lower than that of non-pregnant mice (p<0.001 for all). The percentages of the IL-35-positive cells in the glandular epithelium were significant increase on gestational day 1, 6 and 7 (p<0.009, p<0.018, p<0.001), and obviously higher than that of luminal epithelium and stroma in same gestational day included gestational day 1, 2, 3, 6 and 7. The percentages of the IL-35-positive cells in the stroma showed a significant increase from gestational day 2 to 7 (p<0.001 for all).

The contents of IL-35 in the mouse endometrium in different pregnancy stages

In order to further investigate the pattern of constitutive expression of IL-35 in early pregnancy, we examined the levels of IL-35 in the mouse endometrium in different pregnancy stages by ELISA. As shown in Figure 6, the expression of IL-35 was firstly increased and then decreased.

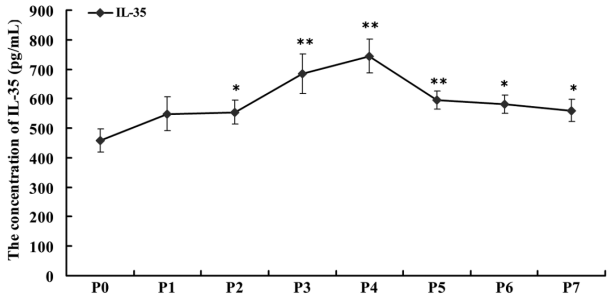

Figure 6 – ***The constitutive expression of IL-35 was detected in the uterus during early pregnancy. The content of IL-35 in the mouse uterus was the highest on gestational day 4 (**p<0.001). The content of IL-35 in the uterus from gestational day 2 to 7 was significantly higher than that of non-pregnant mice (*p< 0.040, **p<0.006 respectively). P0 represented non-pregnancy; P1–P7 represented pregnancy day 1 to 7, respectively. **p<0.01, *p<0.05.***

The level of IL-35 was the lowest in the endometrium of non-pregnant mice, and the significant increase of expression appeared on the second day of pregnancy.

Until the fourth pregnancy day, the expression of IL-35 increased to the highest level. The concentration of IL-35 in the mouse endometrium was significantly 21.04%, 49.54% and 62.58% higher than that of non-pregnant mice from gestational day 2 to 4, respectively (p=0.040, p<0.001 and p<0.001 respectively). However, the expression of IL-35 showed a declining tendency from the 5th day to the 7th day compared to that of the 3rd and 4th days of pregnancy and was remained at a high level, which was still 29.81%, 26.80% and 22.41% higher than that of non-pregnant mice, respectively (p=0.006, p=0.012 and p=0.030, respectively).

Expression of Ebi3 and p35 mRNAs in the mouse endometrium in different pregnancy stages

The above results had confirmed that the expression of IL-35 in the endometrium during early pregnancy was firstly increased and then decreased. We analyzed the mRNA levels of Ebi3 and p35 (two subunits of IL-35) in the endometrium during early pregnancy by qPCR. As shown in Figure 7, the mRNA expression of Ebi3 was rapidly increased from gestational day 2 to 4 compared to that of non-pregnant mice and rapidly decreased from gestational day 5 to 7 compared to that of mice on gestational day 4.

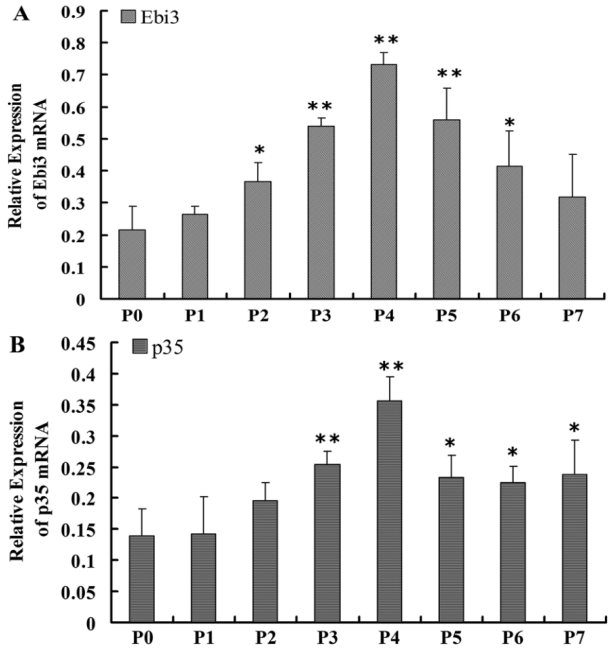

Figure 7 – ***Quantitative real-time PCR was used to analyze the mRNA expression of Ebi3 and p35 in the endometrium during early pregnancy. (A) The expression of Ebi3 mRNA; (B) The expression of p35 mRNA. P0 represented non-pregnancy; P1–P7 represented pregnancy day 1 to 7, respectively. **p<0.01, *p<0.05.***

The expression level of Ebi3 mRNA in the endometrium on gestational day 3 to 5 was respectively 1.5, 2.4 and 1.6 times higher than that of non-pregnant mice (p<0.001, p<0.001 and p<0.001, respectively). The expression level of Ebi3 mRNA in the endometrium on gestational day 2 and 6 was 0.7 and 0.9 times higher than that of non-pregnant mice (p=0.042 and p=0.010, respectively), but the expression of Ebi3 mRNA in the endometrium of non-pregnant mice were not significantly different from those of the mouse on gestational day 1 and 7 (p=0.544 and p=0.150, respectively).

The mRNA expression of p35 in the endometrium during early pregnancy showed similar changes compared to that of Ebi3, but the expression level of p35 mRNA was significantly lower than that of Ebi3. Moreover, the significant increase of p35 mRNA appeared on gestational day 3, and the decrease of p35 mRNA were not found from gestational day 5 to 7. The expression levels of p35 mRNA in the endometrium on gestational day 3 and 4 were 0.8 and 1.5 times higher than that of non-pregnant mice (p=0.004 and p<0.001 respectively), but the increase rate of p35 mRNA was lower than that of Ebi3 on gestational day 3 and 4.

The correlation analysis of different detection items showed that the immunohistochemical expression of IL-35 was significantly correlated with the contents of IL-35 and the expression of Ebi3 mRNA in the mouse endometrium, the correlation coefficients were respectively 0.932 and 0.952 (p=0.001, p<0.001). There were also significant correlation between the contents of IL-35 and the expression of Ebi3 mRNA, the correlation coefficients were respectively 0.922 (p=0.001). However, the correlation coefficients between the expression of p35 mRNA and the immunohistochemical expression of IL-35, the contents of IL-35 and the expression of Ebi3 mRNA were decrease, although the correlations still were significant, the correlation coefficients were respectively 0.910, 0.906 and 0.912 (p=0.002, p=0.002, p=0.002).

Discussion

Successful pregnancy in placental mammals depends upon the establishment and maintenance of maternal-fetal immune tolerance in the uterus. From the perspective of the immune system, pregnancy presents a state akin to organ transplantation, in which fetal antigens are foreign products to the mother's immune system [3]. After being implanting into the endometrium, the expressed paternal antigens triggered complex maternal immune mechanisms, in which maternal immune system only lacked the ability to attack fetal, and still retained other strong immune responses to protect the mother. The establishment of the complex immune mechanisms was one research hotspot in embryo implantation. The leukocytes and cytokines in the maternal-fetal interface, including uterine natural killer cells, macrophages, dendritic cells, CD4+ T-cells, Th1-type cytokines, Th2-type cytokines, etc., participated in the mechanism [33, 34]. Importantly, current reports confirmed that regulatory T-cells were pivotal in the establishment of maternal-fetal immune tolerance compared with other immune cells [24].

Naturally, occurring regulatory T-cells are characterized by the surface expression of CD4 and CD25, which participated in the establishment of immune tolerance against self-antigens and antigens encountered in foreign implantation [35]. The extensive research demonstrated that maternal CD4+CD25+ T-cells are induced by and react against paternal alloantigen, thus contributing to the successful implantation of pregnancy [36–39]. Sasaki *et al.* also observed that suppressive CD4+CD25+ T-cells were enriched in the decidua, and that this enrichment

was not found in the cases of spontaneous abortion [23]. Aluvihare *et al.* further found that the CD4+CD25+ T-cells were significantly increased in all tissues on gestational day 10.5 and that the mRNA expression of specific marker transcription factor forkhead box P3 (Foxp3) of regulatory T-cells were also significantly increased in the uterine tissue [24], indicating that CD4+CD25+ Tregs played a crucial role in the maternal-fetal immune tolerance.

Recent studies have demonstrated that many factors influenced number of CD4+CD25+ Tregs in the process of pregnancy. Estrogen was an important positive signal to induce the proliferation of CD4+CD25+ Tregs *in vivo* [40]. During pregnancy, high concentration of estrogen not only can up-regulate Foxp3 expression level, but also stimulate the IL-10 secretion, further increase the number of CD4+CD25+ Tregs [41]. Secretion of IL-10 induces the expression of IL-10 receptor α and Bcl-2 in CD4+CD25+ Tregs, which lead to an expansion of CD4+ CD25+ Tregs and inhibit apoptosis of CD4+CD25+ Tregs [42, 43]. IDO (Indoleamine 2, 3-dioxygenase) is a rate-limiting enzyme for the essential amino acid tryptophan catabolism and expressed on trophoblastic giant cells in mice and both extravillous trophoblasts and villous trophoblasts in humans [44]. Recently, it was reported that CD4+CD25+ Tregs can up-regulates IDO expression on DCs (dendritic cell) by interaction of CTLA4 and its ligand CD80-CD86 [45, 46], while IDO also can alter function of DCs [47], which further influence the differentiation of CD4+CD25+ Tregs [48]. Trophoblast-derived chemokines have also an important role for maternal-fetal tolerance. It is reported that the presence of CCR4 and its ligand CCL22 produced by macrophage during pregnancy might induce the accumulation of CD4+CD25+high Tregs in the deciduas [49, 50]. Above results indicated that the number of CD4+CD25+ Tregs in the pregnant uterus was obviously influenced by estrogen, IDO and chemokines.

IL-35 was one of immunosuppressive cytokines secreted by non-stimulated mouse CD4+CD25+FoxP3+ Tregs and stimulated human CD4+CD25+FoxP3+ Tregs and the immunomodulatory function of CD4+CD25+FoxP3+ Tregs were completed in an IL-35-dependent manner [25, 30, 51]. Previous studies indicated that IL-35 had an obvious immunosuppressive effect and could directly suppress CD4+ effector cells proliferation *in vitro*, Th17 development *in vivo*, and ameliorate collagen-induced arthritis and that the loss of IL-35 expression resulted in the reduced *in vivo* suppressive capacity of Tregs [52]. IL-35 overexpression can suppress cell growth in human cancer cells and the attenuating effects on allergen-specific CD4+ effector Th2 cell-mediated airway inflammation through inhibiting the secretion of Th2-type cytokines [53, 54]. Furthermore, Mao *et al.* indicated that IL-35 might be an important cytokine of regulating local immune responses in the uterus during human pregnancy [31]. The impact factors (such as estrogen, IDO and chemokines) that influenced the number of CD4+CD25+ Tregs in the maternal uterus might affect the expression of IL-35.

Our findings presented here confirmed the potential effect of IL-35 in the fetal implantation of mouse. In the study, we found that the positive cells, IRS scores and contents of IL-35 in the mouse uterus was firstly increased and then decreased in early pregnancy. The IL-35-positive cells and contents of IL-35was significantly increased on gestational day 2, reached the maximum value on gestational day 4, and then slowly decreased. Moreover, we also found that the distribution of IL-35 in the endometrium was significantly increased from gestational day 2 to 4, and that IL-35- positive cells were significantly increased in the primary decidual zone on gestational day 4. There were significant correlations between the number of IL-35-positive cells, the contents of IL-35 and the expression of Ebi3 mRNA in the uterus. The main reason maybe: (*1*) as embryo implantation and the formation of trophoblast and deciduas, the level of estrogen and progesterone are rapidly increase, which induce secretion of immunosuppressive cytokines (IL-10 and TGF-β) and chemokines (CCR4 and CCL22) and up-regulate IDO expression on DCs, in turn increase number of CD4+ CD25+ FoxP3+ Tregs to promote transcription of Ebi3 and p35 gene and expression of IL-35; (*2*) the placental trophoblasts, stromal cell, decidual cells and other cell in the uterus of pregnant mice also expressed IL-35, which increase the level of IL-35. The current results indicated that IL-35 participated in the regulation of the maternal-fetal tolerance and fetal implantation. In addition, the mRNA expression of Ebi3 subunit of IL-35 was not fully consistent with that of p35 subunit of IL-35, which was significantly higher than that of p35. And the significance of correlation coefficients between the expression of p35 mRNA and the immunohistochemical expression of IL-35, the contents of IL-35 and the expression of Ebi3 mRNA were decrease, which indicating that IL-35 was potentially not constitutively expressed in some cells of maternal-fetal interface.

Moreover, in the study, we also found that the IL-35 positive expression were mainly distributed in luminal epithelium and glandular epithelium from gestational day 1 to 2, which indicate the luminal epithelial cells and glandular epithelial cells mainly participated in the induction of the maternal-fetal tolerance at the beginning stage of gestation. However, as the embryo implantation, the IL-35 positive expression in the stroma were rapidly increased from gestational day 2 to 5, and the IL-35 positive cells in the stroma and glandular epithelium were dominant from gestational day 4 to 7, which indicated glandular epithelial cells and stromal cells mainly regulated the establishment and maintenance of maternal-fetal tolerance for embryonic implantation. Our results showed that the IL-35 played an important role in the regulation of the different cellular subtype in the endometrium for the maternal-fetal tolerance at the different stage of gestation.

Although the mechanism of IL-35-induced maternal-fetal tolerance was not clear, we considered that IL-35 might have the three ways to mediate maternal-fetal tolerance. First, the expansion of CD4+CD25+ Tregs cells was stimulated or effector cells were converted into iTr35 to up-regulate immunosuppressive cytokines IL-10 to mediate maternal-fetal tolerance [30, 41, 55]. Second, trophoblast cells and Th2-cells were promoted to secret inhibitory cytokines (IL-10 and TGF-β) to mediate maternal-fetal tolerance [56]. Third, uterine NK cell function

was affected through a currently unknown way to mediate maternal-fetal tolerance [31]. However, the above ways of IL-35-mediated maternal-fetal tolerance requires further research. Our study provides interesting clues to better understand the mechanisms of maternal-fetal immune tolerance.

Conclusions

Our data reported changes in distribution pattern and constitutive expression of cytokine IL-35 in the mouse uterus during early pregnancy for the first time, indicating that the distribution of IL-35 was enlarged, and the protein and mRNA expression was increased from gestational day 2 to 4. But, the expression of IL-35 was decreased from gestational day 5 to 7.

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Author contribution

Erhui Jin and Chenfang Wang are equal for first author.

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