

## Original Article

# Correlation of TLR4/MyD88 signaling with early miscarriage

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**Abstract:** To study correlation between TLR4/MyD88 signaling and early miscarriage, thirty women with early miscarriage and thirty women with induced absorption after 6- to 10-week pregnancy were enrolled. Chorion and decidual tissues were collected from all patients. TLR4 and MyD88 were detected with immunohistochemical staining and then quantified. Certain cytokines were quantified. HE staining showed that in the presence of early pregnancy loss, trophoblast layer was thinner, cells became flattened, denatured or necrotic and highly acidophilic and fibrosis of connective tissue in villi axis was enhanced in chorion; while in decidua, connections among decidual cells became looser, a part of cells were disassembled or enucleated and cells were highly acidophilic. Serum IL-8 and TNF- $\alpha$  were significantly higher ( $P < 0.01$ ) while IL-6 is significantly lower ( $P < 0.01$ ) in early miscarriage patients. Before treatment, serum IL-8 and TNF- $\alpha$  were significantly higher while IL-6 is significantly lower ( $P < 0.01$ ) in tocolysis patients than those in control group. There was no significant difference among successful and failed tocolysis patients ( $P > 0.05$ ). Serum IL-8 and TNF- $\alpha$  in successful tocolysis patients were lower while IL-6 was higher after than those before treatment ( $P < 0.01$ ) and not significantly different from those in control patients ( $P > 0.05$ ). TLR4 and MyD88 in both chorion and decidual tissue are correlated with occurrence of early miscarriage.

**Keywords:** TLR4, MyD88, early miscarriage, chorion, decidua

## Introduction

Toll-like receptors (TLRs) are involved in innate immunity and bridging innate immunity and acquired immunity [1-3]. They are pattern recognition receptors (PPRs) responsible for pathogen-associated-molecular-patterns (PAMPs) identification, activation of innate immune response such as releasing of inflammatory mediators and eventually activation of acquired immune system [4, 5]. Between mother and fetus, various immune cells and cytokines coordinate with each other in the immune balance. TLR4 expression in placental trophoblast cells induces immune response and leads to releasing of cytokines that are beneficial for resisting pathogenic infection. However, over-stimulated immune response will be harmful and may result in a series of pathological or physiological abnormalities and ultimately lead to miscarriage.

Abnormal TLR4 expression has been found in fetal interface, indicating sustained activation

of signal transduction, releasing of numerous inflammatory factors and serious nonspecific aseptic inflammation that can lead to maternal immune rejection of fetal tissue and miscarriage [6]. Involvement of TLR4 in miscarriage has been implicated in previous studies [7] and lower expression of CD4+CD25+Treg cells in peripheral blood, higher expression of TLR4, as well as the TLR4-NF- $\kappa$ B signaling and genetic variation in TLR4 promoter region might be involved the occurrence of unknown reason spontaneous abortion (URSA). Furthermore, alterations of TNF- $\alpha$ , IL-2, IL-6 and IL-10 in unexplained miscarriage have been reported; on the other hand, LPS could induce placental inflammation and fetal death in normal rather than TLR4-knockout rat, suggesting TLR4 mediates the dyssecretion of inflammatory factors in interface and ultimately, promotes miscarriage [8-12]. All those studies indicated that TLR4 was involved in etiology and progression of miscarriage. Thus, it is interesting to study contribution of TLR4 and related proinflamma-

## Correlation of TLR4/MyD88 with miscarriage

tory cytokines in miscarriage during clinical practice.

Myeloid differentiation protein (MyD88) is an important adaptor protein for TLR4 signaling [13]. It can activate multiple transcription factors and upregulate proinflammatory cytokines and various host defense proteins such as IL-2, IL-6, IL-12, TNF- $\alpha$  and so on. However, its involvement in miscarriage is unclear since previous studies of MyD88 mainly focused on the anti-infection immune responses [14]. Thus it's important to study whether MyD88 signaling mediates the involvement of TLR4 in early miscarriage.

Here, we aimed to study the correlation between TLR4, MyD88 and early miscarriage by comparing expression of TLR4 and MyD88 in chorion and decidua from patients with early miscarriage and normal pregnancy. We also explored the relationship between TLR4/MyD88 signaling and early miscarriage and characterized relevant mechanisms underlying the involvement of TLR4/MyD88 in early miscarriage. Our study will provide insights into the unexplained miscarriage in critical period of fetal development and guides aristogenesis fine rearing.

### Materials and methods

#### *Clinical data*

All subjects were from the Department of Obstetrics and Gynecology of the Second Affiliated Hospital of Inner Mongolia Baotou College and the Baogang Hospital from September 2012 to August 2014. Thirty patients with embryos ceasing development and needing curettage diagnosed with ultrasound screening were included in miscarriage group. Diagnosis of early miscarriage followed the eighth edition of Obstetrics and Gynecology published by the People's Health Publishing House. 30 patients with normal pregnancy (within 12 weeks of gestational age) but voluntarily received abortion were included in control group. All control patients were without history of spontaneous abortion and embryonic development normal under ultrasound screening. After admission, patients in miscarriage group received HCG or progesterone, vitamin E, stayed in bed, kept calm till 28 weeks of pregnancy and then received pharmacies for further treatment.

After successful treatment, patients were discharged and followed up until the date of presumably delivery. All manipulations were approved by Ethic Committee of the Hospital and all patients were well informed and signed written consent.

Criteria for patients inclusion were that both spouses were with normal karyotype, without autoimmune disease or endocrine diseases, without history of recent medication or viral infection, without genital surgery history, without reproductive tract abnormalities, uterine fibroids or other diseases under gynecological and ultrasound examination, without intrauterine device, normal under semen examination and negative for anti-phospholipid antibodies, anti-nuclear antibody and anti-sperm antibody.

Criteria for patients exclusion were that patients were with vaginal bleeding during early pregnancy, with usage history of mifepristone and misoprostol or other similar drugs before curettage, with usage history steroids hormone or similar drugs and with chlamydia or mycoplasma infection in genital tract or suffering from torch or acute and chronic inflammation of genital tract.

#### *Reagents*

Antibodies used were as followed: Rabbit anti-human TLR4 polyclonal antibody (Beijing Boosen Biological Technology Co. Ltd., China) and Rabbit anti-human MyD88 polyclonal antibody (Beijing Boosen Biological Technology Co. Ltd., China). Reagents for immunohistochemical experiments were as followed: SP Kit (Fuzhou Maixin Biotechnology Development Co. Ltd, China) and DAB Chromogenic reagent (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., China). ELISA kits for IL-6, IL-8 and TNF- $\alpha$  were from Shanghai Lianshuo Biological Technology Co. Ltd. (China).

#### *Paraffin embedding and slicing*

After sucked with vacuum aspiration and repeated rinsed with saline, chorion and decidua were separated and immersed in 1:20 Bouin solution for 20~24 h. Dehydration was accomplished by transferring tissue block through 70%, 80%, 95%, 95% alcohol for 8 h, overnight, 2 h and 1 h respectively, followed by 2 changes

## Correlation of TLR4/MyD88 with miscarriage

of 100% alcohol, 1 h each. Tissues were then cleared with xylene twice, 30 min each, immersed in paraffin at 62°C twice, 1 h each and embedded in paraffin block. Embedded tissue blocks were further sectioned at 5 µm with a microtome, floated in 42°C water bath and transferred onto poly-lysine glass slides and allowed to dry at 64°C for 1 h followed by 45°C for 5 h.

### *HE staining*

For HE staining, embedded sections were deparaffinized with xylene twice, 10 minutes each and dehydrated gradually through absolute alcohol twice, 5 min and 2 min each, 95% alcohol for 2 minutes, 80% alcohol for 2 min and 70% alcohol for 2 minutes. After washing briefly with running tap water and distilled water for 1 min each, sections were stained in Harris hematoxylin solution for 5 min and rinsed with running tap water for 1 min. Sections were then differentiated in 1% acid alcohol for several seconds and immersed in water and washed with running tap water for 5 m. For staining, sections were counterstained in 1% eosin-phloxine solution for 5 min and rinsed with tap water for 1 min. After Dehydrating in 70%, 80%, 95% alcohol for several seconds and in absolute alcohol twice, 12 min and 2 min each, sections were permeabilized with xylene twice, 5 minutes each. Finally, sections were mounted in xylene based mounting medium for further imaging.

### *Immunostaining*

Deparaffinized sections (in 2.2) were washed with 0.01 M PBS for three times, 5 min each. Then slides were placed in prepared retrieval buffer and heated in a microwavable container. Microwave was set on high gear until buffer was boiled with little bubbles and then set on low for 10 minutes. Sections were cooled at room temperature and washed with 0.01 M PBS for three times, 5 min each. After wiping PBS solutions, sections were blocked with Reagent A for 30-40 min at room temperature in humid chamber and washed again with 0.01 M PBS for three times, 5 min each. Then sections were blocked for 30 min at room temperature. After Tapping excess serum, sections were incubated in rabbit anti-human TLR4 or rabbit anti-human MyD88 antibody (1:200 in PBS) for 1 h at room temperature in humid chamber

and then overnight at 4°C. On the following day, sections were rinsed with 0.01 M PBS for 3 times, 5 min each and incubated in biotin-conjugated secondary antibody at recommended dilution for 15 min at room temperature in humid chamber. Sections were then washed in 0.01 M PBS for 3 times, 5 min each, incubated in streptavidin biotin-peroxidase solution (Reagent D) at room temperature for 15 min, washed in 0.01 M PBS washing for three times, 5 min each, incubated with DAB substrate solution until fully colored and then immediately placed in distilled water to terminate reaction and immersed in fine stream of water for 15-20 min. Hematoxylin was used for counterstaining. Reverse dehydration was carried out by treating section in 50%, 70% and 95% alcohol for 5 min each and then in 100% alcohol twice, 10 min each. Sections were finally rinsed in xylene twice, 10 minutes each and mounted in neutral resin mounting reagent. Negative control followed same procedures except that PBS instead of primary antibody was added.

### *Enzyme linked immunosorbent assay (ELISA)*

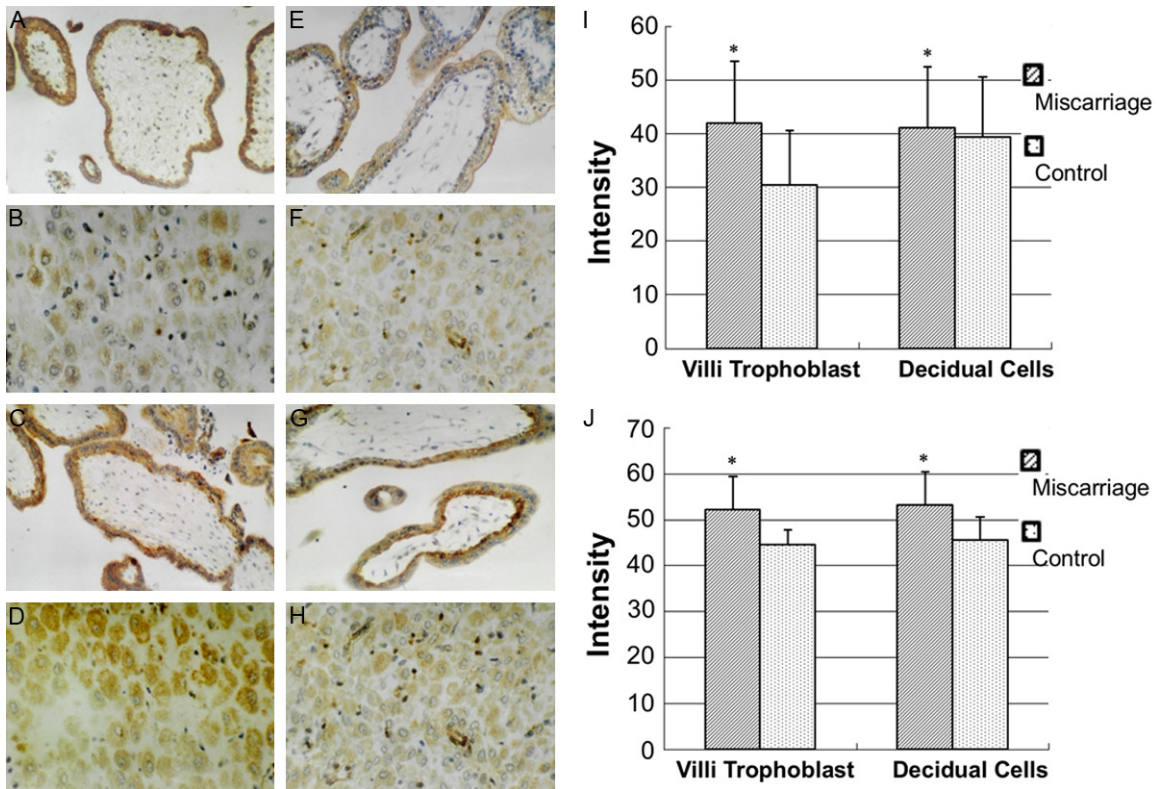
Three-ml elbow venous blood was collected from fasted subjects in morning. After 1 h, blood was centrifuged for 10 min at 2000 rpm. Supernatant serum was collected, separated into 2 tubes and frozen in -20°C. ELISA was used to detect serum IL-6, IL-8 and TNF-α following standard protocols.

Standard curve of ELISA was generated with standard 2500, 1250, 625, 312.5, 156.25, 78.125, 39.06, 0 pg/ml and OD as abscissa in semi logarithmic paper. All OD values were calculated by subtracting value of blank well. Concentration of IL-6, IL-8, TNF-α were calculated based standard curve.

### *Image analysis*

TLR4 and MyD88 in villi and decidua from both control and miscarriage patients were determined after image analysis. 30 cases in each group and 2 repeats in each case from villi and decidua were used for statistical analysis. For each section, 2 regions of interest from trophoblast cells of villi and decidua as well as glandular cells of decidua were randomly selected and measured under optical microscope (200×) by average integral optical density values as relative level of TLR4 and MyD88 (±s), respectively.

## Correlation of TLR4/MyD88 with miscarriage



**Figure 1.** Semiquantification of TLR4 and Myd88 with immunohistochemical staining. TLR4 (A, B, E, F) and MyD88 (C, G, D, H) staining in villi (A, C, E, G) and decidua (B, D, F, H) from miscarriage patients (A-D) and control patients (E-H) (200×). (I and J) represent quantification of signal intensity for TLR4 (I) and MyD88 (J), respectively. \*indicates  $P < 0.05$ , *t* test.

**Table 1.** Quantification of TLR4

Group	Villi Trophoblast	Decidual cells
Miscarriage	42.148±11.376*	41.186±11.388*
Control	30.568±10.125	39.516±11.206
F	44.206	45.670
P	0.026	0.031

\* $P < 0.05$ , *t* test.

**Table 2.** Expression of MyD88

Group	Villi Trophoblast	Decidual cells
Miscarriage	52.268±7.210*	53.372±7.160*
Control	44.706±3.021	45.622±5.021
F	30.046	30.041
P	0.020	0.026

\* $P < 0.05$ , *t* test.

### Statistics

SPSS14.0 software was used for statistical analysis. *t* test was applied to compare difference of TLR and MyD88 in villi and decidua

between control and miscarriage patients, respectively. Pearson correlation analysis was used to analyze correlation between TLR4 and MyD88 in villi and decidua between control and miscarriage patients. After testing homogeneity of variance, levels of IL-6, IL-8, TNF- $\alpha$  were compared in two groups with *t* test and one-way ANOVA with the LSD post-hoc test for multiple-groups comparison. Significance was accepted if  $P < 0.05$ .

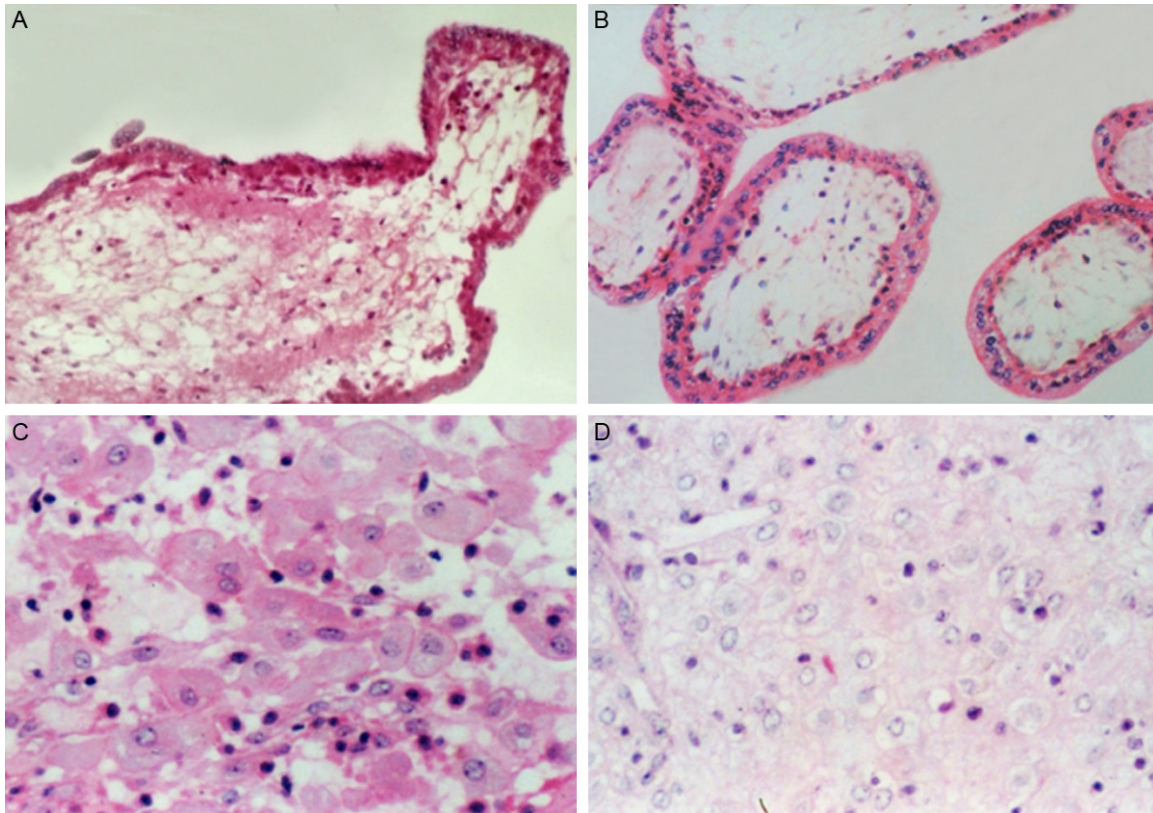
### Results

#### Expression of TLR4 and MyD88 in villi and decidua

TLR4 and MyD88 were visualized as brown yellow or brown signals in trophoblast cells of villi and decidual epithelial cells of decidua. Blue purple indicates the nuclei stained by the hematoxylin. TLR4 (Figure 1A-D, 1I) and MyD88 (Figure 1E-H, 1J) signals were higher in samples from miscarriage patients than control (Figure 1I, 1J; Tables 1, 2), indicating higher



## Correlation of TLR4/MyD88 with miscarriage



**Figure 2.** Hematoxylin-eosin staining in villi (A and B) and decidua (C and D) in miscarriage (A and C) and control group (B and D). 200 $\times$ .

expression of TLR4 and MyD88 in miscarriage patients.

### *Correlation between TLR4 and MyD88 in the villi and the decidua from miscarriage patients*

In the miscarriage patients, expression of TLR4 was positively correlated with expression of MyD88 in both villi ( $r=0.639$ ,  $P < 0.05$ ) and decidua ( $r=0.648$ ,  $P < 0.05$ ).

### *Pathological changes of villi and decidua*

In villi, compared with control (**Figure 2B**), trophoblast layer in miscarriage patients was thinner and cells became flatter, degenerative, necrosis and more eosinophilic and fibrosis of connective tissue in villi axis was also enhanced (**Figure 2A**); while in decidua, compared with lighter color, clearer boundary and denser arrangement of cells in control (**Figure 2D**), decidual cells became looser and part of decidual cells disintegrated, enucleated and more eosinophilic in miscarriage patients (**Figure 2C**).

### *TNF- $\alpha$ , IL-8, IL-6 in serum*

Serum TNF- $\alpha$  and IL-8 were higher while IL-6 was lower in miscarriage patients compared with control ( $P < 0.01$ , **Table 3**).

### *Comparison of serum TNF- $\alpha$ , IL-8 and IL-6 in control with miscarriage patients before tocolysis treatment*

Serum TNF- $\alpha$ , IL-8 and IL-6 were measured in patients with miscarriage before tocolysis treatment. Based on the effects of tocolysis treatment, patients were further subdivided into successful and failed tocolysis groups. Before treatment, there were no significant differences among TNF- $\alpha$ , IL-8 or IL-6 level between two groups ( $P > 0.05$ , **Table 4**). However, in both two groups, serum IL-8 and TNF- $\alpha$  were higher while IL-6 was lower before treatment than those in control ( $P < 0.01$ , **Table 4**), suggesting serum TNF- $\alpha$ , IL-8 and IL-6 are correlated with abnormal pregnancy.

## Correlation of TLR4/MyD88 with miscarriage

**Table 3.** Serum TNF- $\alpha$ , IL-8 and IL-6 in control and miscarriage groups

Group	Cases (n)	TNF- $\alpha$ (pg/ml)	IL-8 (pg/ml)	IL-6 (pg/ml)
Miscarriage	55	326.11 $\pm$ 39.27*	1888.17 $\pm$ 342.53*	20.11 $\pm$ 2.94*
Control	31	169.38 $\pm$ 46.12	656.65 $\pm$ 156.39	123.42 $\pm$ 23.63

\*P < 0.01, t test.

**Table 4.** Serum TNF- $\alpha$ , IL-8 and IL-6 in control and tocolysis groups before treatment

Group	Case (n)	TNF- $\alpha$ (pg/ml)	IL-8 (pg/ml)	IL-6 (pg/ml)
Successful tocolysis	42	312.35 $\pm$ 51.89*	1792.73 $\pm$ 475.83*	21.13 $\pm$ 5.82*
Failed tocolysis	13	375.42 $\pm$ 86.23*	2030.15 $\pm$ 405.75*	16.42 $\pm$ 3.14*
Control	31	169.38 $\pm$ 46.12	656.65 $\pm$ 156.39	123.42 $\pm$ 23.63

\*P < 0.01, one-way ANOVA with the LSD post-hoc test.

**Table 5.** Serum TNF- $\alpha$ , IL-8 and IL-6 before and after successful tocolysis treatment

Group	Case (n)	TNF- $\alpha$ (pg/ml)	IL-8 (pg/ml)	IL-6 (pg/ml)
Before treatment	42	312.35 $\pm$ 51.89	1792.73 $\pm$ 475.83	21.13 $\pm$ 5.82
After treatment	42	182.37 $\pm$ 46.64*	628.45 $\pm$ 201.58*	105.26 $\pm$ 15.79*
Control	31	169.38 $\pm$ 46.12	656.65 $\pm$ 156.39	123.42 $\pm$ 23.63

\*P < 0.01, one-way ANOVA with the LSD post-hoc test.

### *Serum TNF- $\alpha$ , IL-8, IL-6 before and after successful tocolysis treatment*

After successful tocolysis treatment, serum TNF- $\alpha$  and IL-8 were significantly decreased compared with before treatment (P < 0.01, **Table 5**) and restored to control level (P > 0.05, **Table 5**), while serum IL-6 was significantly increased (P < 0.01, **Table 5**) to the level similar to that in control (P > 0.05, **Table 5**).

### **Discussion**

TLR4 is a type I transmembrane protein important for type recognition in innate immune system and has been implicated in spontaneous miscarriage in which it might induce immune imbalance between maternal and fetal and cause pathological changes of maternal fetal interface villi [15]. Therefore, TLR4 might be involved in etiology and development of spontaneous miscarriage in which maternal fetal tissue produces immune rejection to the fetus. In this study, we included clinical sample and demonstrated the positive correlation between miscarriage and TLR4-MyD88 signaling and certain cytokines, our study provide clinical evidence for the involvement of those signaling molecule in etiology and development of spontaneous miscarriage.

A study by Du et al. found that expression of CD4+CD25+Treg is lower while expression of TLR4 is higher in peripheral blood of URSA patients, [10] suggesting the involvement of TLR4 in occurrence of URSA and the presence of maternal fetal aseptic inflammation in spontaneous abortion and the involvement of TLR4 in the pathogenesis of recurrent spontaneous abortion. This was consistent with Yang et al., [16]. In current study, we characterized morphological changes of villi trophoblastic cells, decidual cells and glands and expression of TLR4 and MyD88 in villi and decidua. We found that compared with control, firstly, TLR4 and MyD88 were localized in cytoplasm of villi cells and their expression were higher in miscarriage patients (P < 0.05); secondly, trophoblast layer became thinner, trophoblast cells became flatter, degenerated and more eosinophilic and the fibrosis of connective tissue in villi axis was enhanced, suggesting existing of aseptic inflammation; thirdly, in decidua, TLR4 and MyD88 were localized in the cytoplasm of decidual cells and their expression were higher than the control (P < 0.05); fourthly, decidual cells became loosely connected and part of them were disintegrated, more eosinophilic and enucleated. Together with previous studies [17] our observations showed the occurrence of aseptic inflammation in early miscarriage

## Correlation of TLR4/MyD88 with miscarriage

and suggested an important role of TLR4/MyD88 signaling in pathogenesis and progression of early spontaneous abortion.

We further analyzed the correlation between TLR4 and MyD88 in villi and decidua and they were positively correlated ( $r=0.639$ ;  $r=0.648$ ;  $P < 0.05$ ), indicating involvement of TLR4 may be through MyD88 that stimulates releasing of inflammatory factors such as IL-1/IL-6/IL-8/TNF- $\alpha$  by TLR4- MyD88-NF- or TLR4- MyD88-AP-1 signaling. Such abnormal inflammatory response could result in degeneration and necrosis of maternal fetal interface cells and villi, decidual necrosis and degeneration, inhibition of trophoblastic growth, embryonic development and fetal survival, and abortion. Maternal fetal interface is responsible for resisting invasion of pathogenic microorganisms by producing immune response to such invasion and immune tolerance, so that embryo can be implanted and develop. Thus regulation of immune balance between maternal fetal interfaces is important for normal pregnancy. Destruction of immune balance can results in maternal immune attacking against embryos and abortion.

Our study showed that expression of IL-8 and TNF- $\alpha$  were higher in patients with early spontaneous abortion than those in control and lower in successful tocolysis-treated patients after treatment than before treatment, suggesting IL-8 and TNF- $\alpha$  were involved in occurrence of early spontaneous abortion. IL-6 is secreted by TH2 cells in NK cells of decidua. Previous studies showed that IL-6 could enhance stability of human leukocyte antigen (HLA)-C mRNA in cultured trophoblast cells. Such effect can increase expression of HLA-C in trophoblast cell surface in maternal fetal interface, decrease expression of monocyte I and II class MHC antigens, effectively prevent fetal maternal rejection and is beneficial for normal pregnancy.

All above results indicated that TNF- $\alpha$ , IL-8 and IL-6 are important in reproduction. Before treatment, serum TNF- $\alpha$  and IL-8 were significantly higher while IL-6 was significantly lower in miscarriage patients. 42 out of 55 miscarriage patients were treated successfully with tocolysis. Serum TNF- $\alpha$  and IL-8 were significantly lower in successfully-treated patients than before treatment, while in the 13 patients

failed to be treated, they were still significantly higher than control and serum IL-6 was significantly lower. Those results suggested that expression of TNF- $\alpha$ , IL-8 and IL-6 was correlated with treatment outcome and might serve as markers for early diagnosis of early miscarriage.

In summary, immunological factors are important in early miscarriage. The expression of TLR4 and MyD88 were higher in villi and decidua, indicating their involvement in pathogenesis and progression of early miscarriage. In miscarriage patients, positive correlation between TLR4 and MyD88 indicated the involvement of TLR4 in miscarriage might be executed through MyD88 signaling. However, further studies are still required for dissecting downstream mechanism of TLR4-MyD88 signaling in pathogenesis and progress of early miscarriage.

### Disclosure of conflict of interest

None.

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## Correlation of TLR4/MyD88 with miscarriage

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