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Human Pregnancy Up-Regulates Tim-3 in Innate Immune Cells for Systemic Immunity

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Pregnant women have both the local immune tolerance at the maternal-fetal interface and the systemic immune defense against pathogens. To date, regardless of the extensive investigation on the maternal-fetal immune tolerance, the maintenance of systemic immune defense in pregnant women still remains poorly understood. In the present study, we demonstrate that the immunoregulatory molecule T cell Ig and mucin domain (Tim)-3 plays important roles in innate and adaptive immunity of human pregnancy. During pregnancy, Tim-3 is strikingly up-regulated in peripheral blood of pregnant women, most by monocytes but not by T or B cells. The increased IL-4/STAT6 signaling may contribute to such up-regulation of Tim-3. In turn, the increased Tim-3 enhances not only innate immunity but also Th1-associated immune responses of pregnant women against pathogens. In contrast, our clinical data show that abnormal Tim-3 expression level might be connected to the pregnancy loss. In conclusion, our data show in this study that an immune regulatory molecule Tim-3, by virtue of its up-regulation in innate immune cells in pregnant women, enhances both innate and adaptive immune responses. Nevertheless, the abnormality of Tim-3 in pregnant women may be deleterious to normal pregnancy. The Journal of Immunology, 2009, 182: 6618–6624.

Materials and Methods

Study subject

Forty-two healthy pregnant women were recruited at Tongji Hospital. Blood samplings were obtained at three periods during their pregnancy (10–20, 20–30, and 30–40 wk), after informed consent and with approval of the Clinical Research Ethics Board of the Tongji Medical College, including peripheral blood from normal donors. Pregnant women before the tenth week of gestation were not recruited, because many common infections especially those caused by intracellular microbes depend on Th1-like responses for eradication. In fact, neither viral nor other infections (such as tuberculosis) seem to occur more commonly in pregnancy (13). Therefore, the immune system has adapted strategies to maintain the Th1 responses in pregnancy. In contrast, although the activation of innate immunity during pregnancy has been described in humans (14, 15), the underlying mechanisms remain incompletely understood.

T cell Ig and mucin domain (Tim)-3 is originally identified as a Th1-specific cell surface molecule that down-regulates Th1 responses through transducing apoptosis signaling by galectin-9 engagement (16, 17). These findings suggest that Tim-3 may modulate the Th1/Th2 balance. Moreover, recent reports show that Tim-3 is also expressed on innate immune cells such as dendritic cells and seems to promote innate immunity (18, 19). Therefore, such features of Tim-3 coincide with the paradigm of Th1/Th2 shift and the activation of the innate immune system in pregnancy. In this regard, it is possible for Tim-3 to play a role in systemic immune defense in pregnancy by regulating both innate and adaptive immunity. In this study, we demonstrated that the up-regulation of Tim-3 in innate immune cells is an intrinsic event for human pregnancy by fine tuning the normal innate and adaptive immune defense.

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PBMC were isolated by centrifugation over a Ficoll-Hypaque gradient. The PBMCs were either used directly or cryopreserved in liquid nitrogen.

Isolation of immune cells from peripheral blood

To isolate innate immune cells, PBMCs were labeled with biotin-anti-human CD3 and CD14 Abs (Miltenyi Biotec) and passed through streptavidin-microbeads (Miltenyi Biotec) to get rid of T cells and B cells. After washing, the cells were used for the following experiments. In some cases, the monocytes were separated from PBMCs by CD14 MicroBeads (Miltenyi Biotec). PBMCs were also used for T cell or NK cell isolation by CD3 or CD56 Microbeads (Miltenyi Biotec).

Phagocytosis assay

Escherichia coli, isolated from clinical patient, were cultured to a stationary phase of growth, washed, labeled with fluorescent dye CFSE, and sonized in 10% heat-inactivated serum for 20 min. Suspensions of innate immune cells and CFSE-labeled bacteria were added to incubation tubes at a ratio of 1:10 (cells: bacterial CFU) and incubated for 60 min. Innate immune cells were separated from the unphagocytized bacteria by addition of 100 μg/ml gentamicin during the incubation of second 30 min and analyzed by flow cytometry. The CFSE− cell number and mean fluorescence intensity were used to reflect the phagocytic capacity of innate immune cells.

Bacterial colony counting

One × 10⁶ innate immune cells isolated from PBMCs of five normal pregnant women (20–30 wk) were incubated with E. coli (1 × 10⁹ CFU) in the presence or absence of 1 μg/ml recombinant human Tim-3-Fc fusion (R&D Systems). Thirty minutes later, 100 μg/ml gentamicin was added for another 30 min. The cells were lysed in sterile distilled water and added to sterile brain heart infusion agar plates and incubated at 37°C for 18 h. The colonies were counted.

Flow cytometric analysis

FITC anti-human CD3, allophycocyanin anti-human CD19, PE-Cy7 anti-human CD14, allophycocyanin anti-human CD11b, allophycocyanin anti-human CD356, FITC anti-human CD80, PE anti-human CD83, PE anti-human CD86 Abs, and the corresponding isotypes were purchased from eBioscience. PE anti-human Tim-3 Ab was purchased from R&D Systems. The stained cells were used for flow cytometric analysis (BDTM LSR II, BD Biosciences).

Assay of intracellular H₂O₂ production

Production of H₂O₂ was detected using 2′,7′-dichlorofluorescein diacetate (DCFH-DA), an uncharged, cell permeant fluorescent probe. DCFH-DA was readily diffused into cells, where it is hydrolyzed to the nonfluorescent dichlorofluorescein diacetate (DCFH-DA). DCFH-DA was amplified by PCR to identify the binding site of STAT6. The chromatin was sheared by sonication and purified for immunoprecipitation with an Ab against STAT6. After reversal of cross-linking, DNA was amplified by PCR to identify the binding site of STAT6.
polar derivative DCFH and thereby trapped within the cells. In the presence of H2O2, DCFH is oxidized to the highly fluorescent DCF with excitation and emission of 495 and 525 nm. In this assay, cells were incubated with DCFH-DA (0.5 μM) for 1 h, and then used for flow cytometric analysis.

**ELISA and NO measurement**

The amounts of IFN-γ, IL-4, IL-6, IL-17, IFN-β, and RANTES in subject serum or cultured cell supernatant were determined by ELISA kits from R&D Systems. In addition, NO was measured by Greiss reagent (Sigma-Aldrich).

**Chromatin immunoprecipitation assay (CHIP)**

The experiments were performed using a CHIP assay kit from Upstate Biotechnology according to the manufacturer’s instructions. The relative quantity of mRNA was determined. The resulting data were analyzed with the comparative CT method for relative gene expression quantification against the housekeeping gene GAPDH.

**Proliferation assay**

Two × 10^5 PBMCs isolated from pregnant women (HLA-A*0201, 20–30 wk) or 1 × 10^6 isolated T cells plus 1 × 10^5 irradiated PBMCs were cultured in 96-well round-bottom plates in triplicate, and stimulated with anti-CD3 Ab (1 μg/ml) or 10 μg CMVpp65 peptide (NLVPVMVATV) for 3 days in the presence or absence of Tim-3-Fc fusion (1 μg/ml). To determine the proliferation of T cells, 1.0 μCi [3H]thymidine was added during the last 10 h of culture. The incorporation of [3H]thymidine was measured.

**Cytotoxicity assay**

After 10 days of stimulation with pp65 peptide, the activated T cells were isolated with microbeads and used as effector cells. The MRC-5 fibroblasts (Sigma-Aldrich) were infected with human CMV virus AD169 strain (American Type Culture Collection) at an multiplicity of infection of 10 and used as target cells. A standard 4-h 51Cr release assay was performed. The percent-specific lysis was calculated by the formula: percentage lysis = 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

**Conventional RT-PCR and real-time RT-PCR**

Total RNA was extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The relative quantity of mRNA was determined by RT-PCR (twenty-eight cycles, One-step RT-PCR kit, Qiagen). The mRNA of GAPDH was used as the internal control. The primer sequences were as follows: inducible NO synthase (iNOS), sense 5’-CTCCAGGAAGCAAGATGAG-3’; antisense 5’-TTGTGTGTTGAGTAACGC-3’; IL-6, sense 5’-CCCTTCATTGACCTCAACTAC-3’, antisense 5’-CTCAGCAGATGAGATTCCAAAG-3’; GAPDH, sense 5’-CATGCTACATTGACCTCACAATCTAC-3’, antisense 5’-GGTTGATGGGATTCCATTGG-3’.

For real-time RT-PCR assays, the cDNA sequences of all detected genes were retrieved from the NCBI database. The primers were designed with Oligo Primer Analysis 4.0 software and the sequences were blasted. One hundred nanograms of total RNA was used for reverse transcription using Superscript II RNase H reverse transcriptase (Invitrogen) in a volume of 25 μl. Then 2 μl of cDNA was amplified with SYBR Green Universal PCR Mastermix (Bio-Rad) in duplicate. For sample analysis, the threshold was set based on the exponential phase of products, and CT value for samples was determined. The resulting data were analyzed with the comparative CT method for relative gene expression quantification against the housekeeping gene GAPDH.

**Statistics**

Results were expressed as mean value ± SD and interpreted by ANOVA test. Differences were considered to be statistically significant when p < 0.05.

**Results**

**Up-regulation of Tim-3 in pregnant women**

We examined the expression of Tim-3 in PBMCs of healthy pregnant and nonpregnant women. The real time RT-PCR result revealed that the mRNA level of Tim-3 was increased throughout pregnancy at 10–20, 20–30, and 30–40 wk of gestation, respectively (Fig. 1A). Consistently, the flow cytometric analysis showed
that Tim-3+ cells were significantly increased in CD3− but not CD3+ PBMCs (Fig. 1B). Further analysis showed that Tim-3 was not expressed by CD19+ cells (Fig. 1C), nevertheless, 68.4% Tim-3+ cells coexpressed CD11b and CD14, markers for monocytes (Fig. 1D). In addition, some CD3+ CD56+ NK cells (7.8%) could express Tim-3 (Fig. 1E). Thus, Tim-3 expression was increased in innate immune cells, most by monocytes, during pregnancy.

**IL-4/STAT6 signaling contributes to Tim-3 up-regulation in pregnant women**

Next, we investigated the possible molecular mechanism of up-regulating Tim-3 expression in monocytes of pregnant women. We screened the sequence of Tim-3 promoter using Gene2Promoter software (Genomatix Software). Two binding sites for STAT6, the downstream molecule of IL-4 signaling pathway (20), were found. Coincidently, we also observed the increased IL-4 level in pregnant woman (Fig. 2A). These caused us to test whether Tim-3 is up-regulated by IL-4 signaling in monocytes. By IL-4 stimulation, Tim-3, at both mRNA and protein levels, were significantly increased in monocytes of nonpregnant normal women (Fig. 2, B and C). Consistently, the activation of STAT6 (phosphorylation) was confirmed by Western blot (Fig. 2D). Such activated STAT6 could directly bind to the concordant sequence of Tim-3 promoter, evaluated by a CHIP assay (Fig. 2E).

Taken the antagonist effect of IFN-γ on IL-4, in this study, we additionally tested the effect of IFN-γ on Tim-3 expression. The result showed that IFN-γ attenuated the effect of IL-4 on Tim-3 expression in monocytes (Fig. 2, B and C), concomitant with inhibiting the activation of STAT6 by IL-4 (Fig. 2D). Moreover, in contrast to IL-4 level, the blood level of IFN-γ is decreased in pregnant woman (Fig. 2A). Therefore, we identified that Tim-3 expression in monocytes was regulated by IL-4 and IFN-γ. The shift of these two cytokines might explain the increased Tim-3 in pregnancy.

**Tim-3 signaling interception impairs innate immunity in pregnant women**

To explore the immunological relevance of the increase of Tim-3, we hypothesized that the up-regulation of Tim-3 augmented innate immunity of pregnant woman. To test this, we chose innate immune cells rather than sole monocytes of pregnant women for the in vitro phagocytosis assay. We labeled E. coli bacteria with CFSE and incubated them with innate immune cells in the presence or absence of recombinant human extracellular Tim-3-Fc fusion to intercept Tim-3 signaling. In the absence of Tim-3-Fc fusion, cells could effectively ingest bacteria, evaluated by the mean fluorescence intensity, although the number of CFSE+ cells was not obviously changed (Fig. 3, A and B); the effective bactericide was also observed by bacterial colony counting (Fig. 3C). However, in the presence of Tim-3-Fc fusion, the ingestion and bactericidal activity of cells were significantly decreased (Fig. 3, A–C). In line with these data, the amount of intracellular hydrogen peroxide (H₂O₂) and the mRNA level of iNOS and NO in supernatant were significantly decreased after Tim-3 blockade (Fig. 3, D–F). Taken together, these data suggested that the increase of Tim-3 may strengthen innate immunity of pregnancy, leading to effective defense against infections.

**Tim-3 signaling interception attenuates LPS-triggered TLR signaling**

To further elucidate the role of Tim-3 in innate immunity in pregnant woman, we asked whether Tim-3 affected TLR signaling, the central player in innate immunity. To test this, innate immune cells of pregnant women were stimulated with 100 ng/ml LPS in the presence or absence of Tim-3-Fc fusion. LPS stimulation effectively activated TLR signaling, evaluated by the increased transcription activity of NF-κB and AP-1 in LPS-stimulated innate immune cells of pregnancy. One × 10⁶ innate immune cells isolated from PBMCs of six normal pregnant women were separately stimulated with LPS (100 ng/ml) in the presence or absence of Tim-3-Fc fusion for 6 h. The activities of NF-κB (A) and AP-1 (B) were assayed as described in Materials and Methods. *, p < 0.05, compared with bacteria group. C–E, Detection of IL-6 and iNOS. One × 10⁶ innate immune cells were stimulated with LPS (100 ng/ml) in the presence or absence of Tim-3-Fc fusion. Six hours later, IL-6 and iNOS mRNAs were analyzed by RT-PCR (C); 48 h later, the productions of IL-6 (D) and NO (E) in the supernatant were measured by ELISA and Greiss reagent, respectively. *, p < 0.05, compared with bacteria group. F and G, Tim-3 blockade did not affect TRIF-dependent TLR signaling after LPS stimulation. The PBMCs were pretreated with 100 μM MyD88 inhibitory peptide (Imgenex). Twelve hours later, LPS, Tim-3-Fc fusion, and additional 100 μM MyD88 inhibitory peptide were added to the culture medium. Forty-eight hours later, the production of IFN-β (F) and RANTES (G) in the supernatant was detected by ELISA.

**FIGURE 4.** Tim-3 blockade attenuates TLR signaling triggered by LPS. A and B, Tim-3 blockade decreased the transcriptional activities of NF-κB and AP-1 in LPS-stimulated innate immune cells of pregnancy. One × 10⁶ innate immune cells isolated from PBMCs of six normal pregnant women were separately stimulated with LPS (100 ng/ml) in the presence or absence of Tim-3-Fc fusion for 6 h. The activities of NF-κB (A) and AP-1 (B) were assayed as described in Materials and Methods. *, p < 0.05, compared with bacteria group. C–E, Detection of IL-6 and iNOS. One × 10⁶ innate immune cells were stimulated with LPS (100 ng/ml) in the presence or absence of Tim-3-Fc fusion. Six hours later, IL-6 and iNOS mRNAs were analyzed by RT-PCR (C); 48 h later, the productions of IL-6 (D) and NO (E) in the supernatant were measured by ELISA and Greiss reagent, respectively. *, p < 0.05, compared with bacteria group. F and G, Tim-3 blockade did not affect TRIF-dependent TLR signaling after LPS stimulation. The PBMCs were pretreated with 100 μM MyD88 inhibitory peptide (Imgenex). Twelve hours later, LPS, Tim-3-Fc fusion, and additional 100 μM MyD88 inhibitory peptide were added to the culture medium. Forty-eight hours later, the production of IFN-β (F) and RANTES (G) in the supernatant was detected by ELISA.
In this study, we further determined the possible downstream signaling of TLR affected by Tim-3. We used MyD88 inhibitory peptide (Imgenex Corp) to interfere with MyD88 homodimerization. Under such condition, we found that Tim-3 blockade did not influence the production of RANTES and IFN-γ/H9252, the TRIF-dependent cytokines (22) (Fig. 4, F and G). Therefore, these data suggested that Tim-3 blockade may blunt TLR signaling through MyD88-dependent signaling pathway.

**Tim-3 signaling interception impedes adaptive immunity in pregnant women**

Considering that TLR signaling may serve as the bridge between innate and adaptive immune responses, we hypothesized that Tim-3 played a role in adaptive immune responses in pregnant woman. To test this, PBMCs of pregnant women were stimulated with anti-CD3 Ab in the presence or absence of Tim-3-Fc fusion. The result showed that the polyclonal T cell proliferation was inhibited by Tim-3 blockade (46.3 ± 7.2% inhibition, Fig. 5A), concomitant with decreased IFN-γ secretion (Fig. 5B). In parallel, Tim-3 signaling interception also inhibited specific T cell proliferation and IFN-γ production induced by human cytomegalovirus pp65-derived peptide (Fig. 5, A and B). However, IL-17, an important proinflammatory cytokine to which Tim-3 has been associated (23), seemed not to be affected by Tim-3 blockade (Fig. 5C).

For these data, we concerned the contamination by other cells of PBMCs. In this regard, we isolated T cells from PBMCs and cultured them with irradiated PBMCs under the stimulation of anti-CD3 Ab or pp65 peptide. Consistently, Tim-3 blockade also impaired T cell proliferation (Fig. 5A) and IFN-γ production (Fig. 5B). Further analysis revealed that Tim-3 blockade decreased the expression of surface molecules CD80, CD83, and CD86 in PBMCs after pp65 peptide stimulation (Fig. 5D). We then determined the cytotoxicity of T cells. The pp65 peptide-induced T cells and cytomegalovirus-infected fibroblasts were used as effector cells and target cells, respectively. The result showed that the cytotoxicity was significantly decreased in Tim-3-Fc fusion group (Fig. 5E). In this study, we additionally tested whether Tim-3 blockade affected the killing activity of NK cells, considering the important role of NK cells in the maternal-fetal interface. The isolated NK cells were cultured with IL-2 (1,000 U/ml) and Tim-3-Fc fusion for 20 h. Then the NK cells were used as effector cells. KS62 cells or HCMV-infected fibroblasts were used as target cells. The cytotoxicity was assayed under the ratio of E:T = 10:1.

**FIGURE 6.** Tim-3 abnormality in late miscarriage. A and B, Tim-3 expression in late miscarriage. PBMCs were isolated from 17 patients with miscarriage (12–20 wk), and used for Tim-3 detection by real time RT-PCR (A) or flow cytometric analysis of Tim-3 (B). Among them, eight patients overexpressed Tim-3 and four patients extremely lowly expressed Tim-3. The normal pregnant (10–20 wk) and nonpregnant controls were derived from Fig. 1. C, The relationship of Tim-3 expression and the ratio of serum IFN-γ/IL-4. In the above 17 patients, five patients with high level of Tim-3 had decreased IFN-γ/IL-4 ratio.
cells in pregnancy and the expression of Tim-3 by NK cells (Fig. 1). A NK-sensitive human leukemia cell line K562 and CMV-infected fibroblasts were used in this study. The result showed that Tim-3 blockade had no significant influence on NK cell cytosis to either K562 cells or CMV-infected cells (Fig. 5F).

A relationship between Tim-3 abnormality and late miscarriage

To explore the possible clinical significance of Tim-3, we analyzed 17 blood samples from patients with late miscarriage (12–20 wk). The result by real time RT-PCR showed much lower Tim-3 in four patients and extremely higher Tim-3 in eight patients, compared with that in normal nonpregnant and pregnant subjects respectively (Fig. 6A). Consistently, the flow cytometric analysis showed a similar result (Fig. 6B), suggesting that Tim-3 abnormality exists in some patients. The expression of Tim-3 could be regulated by IL-4 and IFN-γ in an antagonistic manner (Fig. 2), we therefore determined IL-4 and IFN-γ in the miscarriage patients. Measurement of the levels of IFN-γ and IL-4 in patients’ blood further disclosed that a decreased IFN-γ/IL-4 ratio in five subjects was observed in Tim-3-overexpressing eight patients (Fig. 6C). Nevertheless, there was no obviously alteration of IFN-γ/IL-4 ratio in other subjects. Taken together, these data implied that Tim-3 abnormality may possibly at least connect to the late spontaneous abortion.

Discussion

Human PBMC, encompassing a diverse repertoire of both innate and adaptive immune functions, have well-established roles in surveillance for pathogens by means of generated signaling molecules (24). In the present study, we identified that Tim-3 is such a signaling molecule, which plays important roles in innate and adaptive immunity of human pregnancy.

There are three TIM genes in humans. Mature human Tim-3 consists of a 181 amino acids extracellular domain, a 21 aa transmembrane segment, and a 78 aa cytoplasmic tail (25). Tim-3 can be expressed on the surface of activated Th1 cells and triggers the apoptosis pathway of Th1 cells by binding galectin-9 by Tim-3 extracellular domain (17). However, whether Tim-3 on human innate cells also functions as an inhibitory receptor is unclear. In our present and previous studies, we provide evidence that Tim-3 signaling interception by the extracellular domain generated an immunosuppressive consequence, both in human and mouse (26). This phenomenon cannot be explained by the known negative regulation paradigm of galectin 9/Tim-3. Based on our findings and others (19), we propose that Tim-3 might function as a positive receptor for an unidentified ligand or as a ligand for a positive signaling in innate immune cells. Therefore, Tim-3 probably functions as a pivotal immunoregulatory molecule.

During pregnancy, to protect fetus from immunological attack by maternal CTLs, a Th2 bias may be generated with the increased IL-4 level and decreased IFN-γ level in the blood (27, 28). However, attenuation of Th1 immunity by increased Th2 cytokines may also be deleterious to the fetus, owing to the impairment of the defense against Th1-related pathogens. For this discrepancy, our present study may provide an explanation. Regardless of the effect of IL-4 on Th2 induction, increase of IL-4 may up-regulate Tim-3 expression. The latter, nevertheless, potentiates the Th1 responses thus to keep Th1/Th2 balance within the normal range. Such regulatory machinery of Tim-3 expression in pregnancy well annotates the reciprocal conversion of Yin and Yang in immune system under a certain condition. In this regard, we also tested the effect of progesterone and hCG on Tim-3 expression. Both of them could affect the expression of Tim-3 (our unpublished data). Therefore, Tim-3 expression may be regulated by multiple elements in pregnancy.

One interesting finding in the present study is that pregnancy seems not to affect Tim-3 expression by T cells and keep a very low frequency in T cells (Fig. 1). This may be explained by 1) Tim-3 expressed by primed Th1 cells can mediate self apoptosis (17, 29); and 2) various immunoregulatory elements are increased in pregnancy (30–32), which may lead to hypoimmune responses and Tim-3 down-regulation in T cells. In our study, we found that Th2 cytokote IL-4 promotes innate immune cells expressing Tim-3 through STAT6 signaling pathway. Nevertheless, why IL-4 does not promote Tim-3 expression in Th2 cells, if considering IL-4/STAT6-mediated Th2 cell differentiation? One explanation might be that Th2 cells also harbor other pathway(s) to counteract the effect of STAT6-mediated Tim-3 up-regulation, but such STAT6-counteracting pathway(s) is absent in innate immune cells. Another reason might be that the absence of Tim-3 by Th2 cells is beneficial to Th2 bias by avoidance of Tim-3-transduced apoptotic signaling (17).

Up-regulation of Tim-3 in innate immune cells may serve as a regulator for both innate and adaptive immunity during pregnancy. In this study, we showed that Tim-3 enhanced phagocytes to uptake CFSE-labeled bacteria and also facilitated the production of H2O2 and NO for the clearance of invaded pathogens. Such effect of Tim-3 on infection probably is realized by the synergies of Tim-3 with TLR signaling, at least in TLR4 signaling confirmed here. Thus, Tim-3 may enhance innate immunity against pathogens. Moreover, Tim-3 also plays a role in adaptive immunity through improving the function of APCs. In this study, our data clearly indicate that Tim-3 enhances the cytotoxicity of T cells to CMV-infected cells, a common infection in pregnant women (33). Thus, although pregnancy chooses a Th2 bias to avoid immune rejection of fetus, it adapts a strategy by up-regulation of Tim-3 to keep the potential Th1 surveillance against Th1-related pathogens.

Although our present data show that Tim-3 blockade may impair the phagocytosis of innate immune cells of pregnancy, its molecular basis remains unclear. Because TLR signaling can effectively induce the activation of phagocytes, it probably can also enhance the phagocytosis of phagocytes. In support of this idea, a new report shows that MyD88-dependent TLR signaling may regulate SR-A-dependent phagocytosis of bacteria (34). In addition, TLR signaling may regulate the autophagy of innate immune cells, whereas autophagy affects the phagocytic process (35–37). Therefore, TLR signaling might indirectly regulate phagocytosis by effecting on autophagy. Our data in Fig. 4 show that Tim-3 regulates MyD88-dependent signaling pathway of TLR. Therefore, Tim-3 probably affects the phagocytosis of bacteria through regulating TLR signaling pathway.

The role of Tim-3 in the maintenance of systemic immunity of pregnant woman implies that Tim-3 abnormality may jeopardize the normal pregnant process. Our findings support this possibility. In 17 late miscarriage patients, we found that four patients expressed much low level of Tim-3, and eight patients expressed much high level of Tim-3. Such small-scale sample analysis indicates that Tim-3 abnormality seems to connect to the loss of fetus. The partial reason might be that the low level of Tim-3 results in hypoimmune responses and connects to infection; the high level of Tim-3 may results in hyperimmune responses and connects to immune rejection of the fetus. Interestingly, five of the above eight patients with high Tim-3 level presented decreased IFN-γ/IL-4 ratio, but the other three presented unchanged IFN-γ/IL-4 ratio, compared with control. Such inconsistence may be ascribed to that Tim-3 in innate immune cells is also regulated by other factors, such as pregnancy-derived progesterone or hCG (our unpublished
data). Therefore, a multiple-regulation mechanism of Tim-3 expression exists in pregnancy. As a result, the proper Tim-3 level is produced so to facilitate the normal gestation process.

In summary, our data show in this study that an immune molecule Tim-3, by virtue of its up-regulation in innate immune cells in pregnant women, enhances both innate and adaptive immune responses. Nevertheless, the abnormality of Tim-3 in pregnant women may be deleterious to normal pregnancy. Therefore, Tim-3 may be a potential indicator to predict the risk of abortion in pregnant women.

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Disclosures
The authors have no financial conflict of interest.

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UP-REGULATION OF Tim-3 IN HUMAN PREGNANCY