

Relationship of SOCS3 and TGF- β with IDO expression in early pregnancy chorionic villi and decidua

WEI LIU^{1,2*}, YONGLI HUANG^{1*}, GUANYOU HUANG¹, CONGRONG ZHOU¹, XIAOLING ZENG¹, SHUYUN ZHAO¹, LINGFEI WU³, HUA ZHOU¹, QINGQING WU⁴ and LUJUN DAI⁵

¹Reproductive Medicine Center, Department of Obstetrics and Gynecology, Affiliated Hospital of Guizhou Medical University, Guiyang, Guizhou 550004; ²Reproductive Center, People's Hospital of Guizhou Province, Guiyang, Guizhou 550003; ³Department of Gastroenterology, The Second Affiliated Hospital, Shantou University Medical College, Shantou, Guangdong 515041; ⁴Central Laboratory, Affiliated Hospital of Guizhou Medical University; ⁵Department of Pathology, Affiliated Hospital of Guizhou Medical University, Guiyang, Guizhou 550004, P.R. China

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Abstract. We aimed to investigate the expression of suppressors cytokine signaling (SOCS)-3, transforming growth factor (TGF)- β and indoleamine 2,3-dioxygenase (IDO) and to analyse the relationship of SOCS3 and TGF- β with IDO expression in early pregnancy chorionic villi and decidua in the maternal-fetal interface. Western blot analysis and immunohistochemical method were used to detect the expression of TGF- β , SOCS3 and IDO in chorionic villi and decidua tissues of normal pregnant women. SOCS3, TGF- β and IDO protein was identified in chorionic villi and decidua tissues of normal pregnant women and there was a negative correlation between the expression of IDO and SOCS3, but TGF- β expression was positively correlated with IDO expression. The levels of IDO expression in the decidua from normal pregnancies were significantly higher than those in chorionic villi, while the expression of SOCS3 was no significant difference between decidua and chorionic villi. In normal physiological state of pregnancy, SOCS3 and TGF- β may be involved in the regulation of immune tolerance by positive or negative regulation of IDO expression at maternal fetal interface.

Introduction

It is still not fully understood how a fetus escapes from being attacked by the maternal immune system. Previous studies

have suggested that the enzyme indoleamine 2,3-dioxygenase (IDO) is a key protein in the maintenance of maternal-fetal tolerance. During pregnancy, IDO is secreted by placental trophoblast, decidual cells and maternal monocyte-macrophages, which inhibit the T-lymphocyte reaction and mediate the immune tolerance to the fetus via tryptophan depletion and defective tryptophan catabolism (1). Recent studies have mainly focused on the expression and function of IDO. Here, we studied the relationship of suppressor of cytokine signaling (SOCS)-3 and transforming growth factor (TGF)- β with IDO expression in chorionic villi and decidua during pregnancy to investigate the possibility that the two proteins may regulate the expression of IDO at the maternal-fetal interface.

SOCS3 is part of a protein family that binds cytokine receptors, thereby suppressing cytokine signaling. SOCS3 is an essential regulator of leukemia inhibitory factor receptor signaling in trophoblast differentiation. The expression of SOCS3 is decreased in the placentas of women with intrahepatic cholestasis, demonstrating its essential role in placental development (2,3).

TGF- β is abundantly expressed in the endometrium, epithelial glands and trophoblasts. It plays an important role in endometrial inflammatory events associated with menstruation and repair in preparation for implantation, particularly in promoting the decidualization of endometrial stroma, the maternal support of embryo development, immunomodulation at the maternal-fetal interface and the maintenance of normal pregnancy. TGF- β deficiency can lead to miscarriage or fetal death (4,5).

Studies have shown that, when cells were transfected with IDO, SOCS3 can downregulate the expression of IDO, while the expression of IDO can be upregulated in cultured dendritic cells or plasmacytoid dendritic cells (pDCs) to which TGF- β had been added (6). Since IDO, SOCS3 and TGF- β are expressed at the maternal-fetal interface, the aim of this study was to analyze the expression and possible correlation of the three proteins in chorionic villi and decidua.

Correspondence to: Dr Guanyou Huang or Dr Congrong Zhou, Reproductive Medicine Center, Department of Obstetrics and Gynecology, Affiliated Hospital of Guizhou Medical University, 28 Guiyi Street, Guiyang, Guizhou 550004, P.R. China
E-mail: guanyouhuang@hotmail.com; hvv8sz@163.com
E-mail: zhcr1234@126.com

*Contributed equally

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Materials and methods

Ethics statement. This study was conducted according to the principles expressed in the Declaration of Helsinki. The

study was approved by the Institutional Review Board of the Affiliated Hospital of Guizhou Medical University (Guizhou, China). All patients provided written informed consent for the collection of samples and subsequent analysis.

Patients. Twelve normal pregnant women investigated in the study were selected from those who underwent legal termination at the Affiliated Hospital of Guizhou Medical University, Guiyang, between December 2014 and May 2015. This study was approved by the Ethics Committee of Affiliated Hospital of Guizhou Medical University. Signed written informed consents were obtained from the patients and/or guardians. Age, 27.33 ± 3.37 years; gestational age of the subjects, 62.42 ± 6.30 days. Of all the women, normal embryonic development was revealed by ultrasonic examination and those with abnormal reproductive and history of chronic diseases associated with chronic hypertension, kidney disease and diabetes were excluded. Aseptic collection of chorionic villi and decidua (around 100 mg each) was followed by washing with phosphate-buffered saline (PBS) to remove red blood cells. Tissue samples were either preserved at -80°C prior to western blot analysis or immersed in 10% neutral formalin to fix them prior to immunohistochemical assay.

Western blot analysis. Tissue stored at -80°C was placed in a mortar containing liquid nitrogen and ground to powder, after which radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck & Co., Inc., Whitehouse Station, NJ, USA) was added with protease inhibitors (P8340; Sigma-Aldrich; USA Merck & Co., Inc.). The lysate was centrifuged twice at 4°C for 30 min at $18,514 \times g$ and the supernatants were collected. A bicinchoninic acid assay, protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) was used to determine protein content separately. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% gels to separate the proteins, and gels were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (PerkinElmer, Inc., Waltham, MA, USA) by wet transfer method at 120 V (200 mA) for 1 h and 50 min. PVDF membranes were blocked in blocking buffer (Beyotime Institute of Biotechnology) overnight at 4°C . Blots were incubated with primary antibody (rabbit anti-human IDO, SOCS3 and TGF- β polyclonal antibody; 1:1,500; cat. nos. 12006, 2932 and 3711, respectively; Cell Signaling Technology, Inc., Danvers, MA, USA) or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (1:6,000; cat. no. GTX100118; GeneTex, Inc., Irvine, CA, USA) for 3.5 h at room temperature, then rinsed with tris-buffered saline with Tween-20 and incubated with secondary horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G antibody (PerkinElmer, Inc.). Protein bands were visualized by using the ECL kit followed by autoradiography.

To determine whether SOCS3 and TGF- β expression were related to that of IDO, equal amounts of cell lysate extracted from chorionic villi and decidua were transferred to PVDF membrane (PerkinElmer, Inc.) and then checked with IDO polyclonal antibody (1:1,500), following which the membrane was washed with stripping buffer (Beyotime Institute of Biotechnology) and incubated with SOCS3, TGF- β or GAPDH primary antibody (1:500).

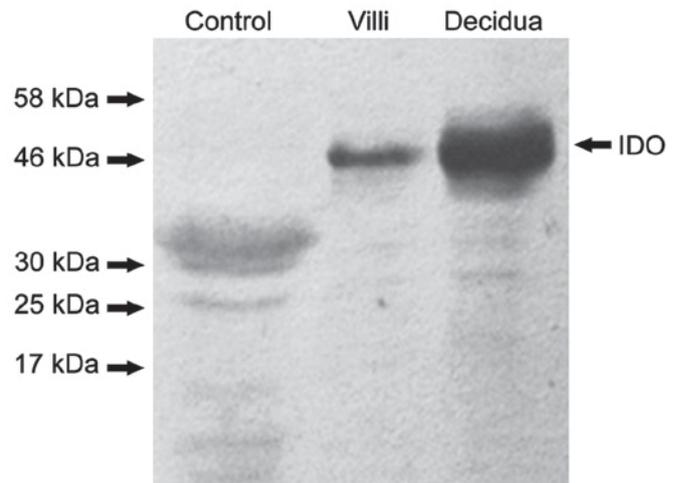


Figure 1. The expression of IDO in chorionic villi and decidua in the negative control. Data are representative of three independent experiments. IDO, indoleamine 2,3-dioxygenase.

Immunohistochemistry. Tissue specimens were embedded in paraffin wax and each sample continuously sliced into $5 \mu\text{m}$ sections. After dewaxing and rehydration, slides were immersed in ethylenediaminetetraacetic acid solution and boiled in an electric pressure cooker (3 min) for antigen retrieval. Cooling was performed at room temperature and endogenous peroxidase activity was quenched with 3% hydrogen peroxide (H_2O_2). Sections were washed with 0.1 MPBS (pH 7.4) between each step of the immunostaining process. The primary antibody (mouse anti-human IDO, SOCS3, and TGF- β monoclonal antibody; cat. nos. ab55305, ab14939 and ab64715, respectively; Abcam, Cambridge, UK) were diluted with antibody dilution buffer (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) before use (dilution concentration of IDO, SOCS3 and TGF- β antibody was 1:200, 1:120 and 1:200, respectively) then incubated overnight at 4°C . Sections were then incubated with rabbit anti-mouse HRP antibody (1:1,000; cat. no. K5007; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 min at 37°C . After being washed three times with PBS (pH 7.4), 3,3'-diaminobenzidine (DAB) solution from a DAB kit (Dako; Agilent Technologies, Inc.) was used as the chromogen. Slides were counterstained with hematoxylin and dehydrated. Thereafter, sections were mounted with coverslips. Immunohistochemical images were evaluated using an Olympus microscope (Olympus, Tokyo, Japan). The scores of immunohistochemical images were defined as -, +, ++ or +++ if 0-9, 10-24, 25-50 or $>50\%$ of the cells were stained positively, respectively. Immunostaining was scored blindly by two independent observers with experience in immunohistochemical pathology.

Statistical analysis. All statistical analyses were performed using the SPSS statistical software package, version 13.0 (SPSS, Inc., Chicago, IL, USA). Pearson's correlation analysis and a paired samples t-test was used to analyze protein expression from western blot analysis and the Chi-square test was used to analyze the results of immunohistochemistry. $P < 0.05$ was considered to indicate a statistically significant analysis.

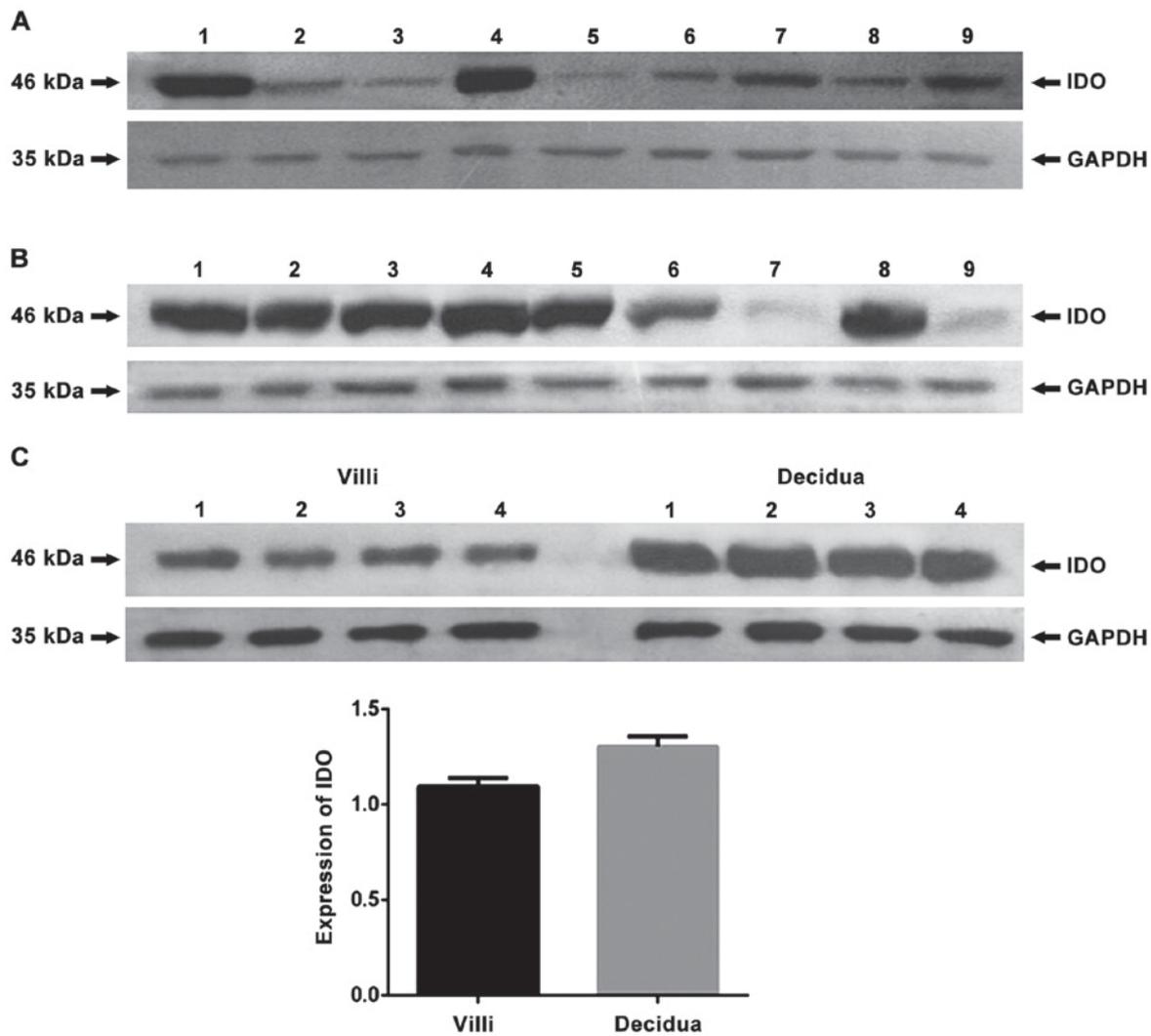


Figure 2. (A) Expression of IDO in chorionic villi and (B) decidua. (C) Comparison of the difference in expression of IDO between chorionic villi and decidua. Labels 1-9 represent different samples of the patients. Data are representative of three independent experiments. IDO, indoleamine 2,3-dioxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Results

Analysis of IDO, SOCS3 and TGF-β expression in chorionic villi and decidua by western blot analysis. To detect the expression of IDO, SOCS3 and TGF-β in chorionic villi and decidua of women in early pregnancy, equal amounts of cell lysates were loaded onto an SDS-PAGE gel, followed by western blot analysis with IDO, SOCS3 and TGF-β polyclonal antibodies or rabbit anti-GAPDH polyclonal antibody. The results showed that, in human peripheral blood mononuclear cells (PBMC), negative control, no protein bands of IDO were found (Fig. 1) and that IDO, SOCS3 and TGF-β were expressed in chorionic villi (Figs. 2A-4A) and decidua (Figs. 2B-4B). Expression of IDO was stronger in the decidua than in chorionic villi (Fig. 2C, P=0.005). No significant difference in SOCS3 expression was observed between chorionic villi and decidua (Fig. 3C, P=0.993), while similar amounts of GAPDH were detected.

In examining whether SOCS3 and TGF-β expression were related to that of IDO, we found that the expression of SOCS3 and IDO were statistically negatively correlated (chorionic

villi, r=-0.808, P=0.004; decidua, r=-0.841, P=0.005), while that of TGF-β and IDO were positively correlated (chorionic villi, r=0.690, P=0.039; decidua, r=0.952, P<0.05) (Figs. 5 and 6).

Analysis of IDO, SOCS3 and TGF-β expression in chorionic villi and decidua by immunohistochemistry. Immunocytochemical staining demonstrated that IDO, SOCS3 and TGF-β are expressed in chorionic villi and decidua. Results of immunohistochemistry were examined at x400 and H&E staining results were examined at x100 magnification. Several representative photomicrographs are shown in Fig. 7. Based on a semiquantitative scoring system, the expression of IDO and SOCS3 were negatively correlated (Table I), but there was a positive correlation between IDO and TGF-β (Table II).

Discussion

Studies have found that IDO-dependent depletion of L-tryptophan decreases the number of cytotoxic T-cells, natural killer (NK) cells and tryptophan metabolites. In particular,

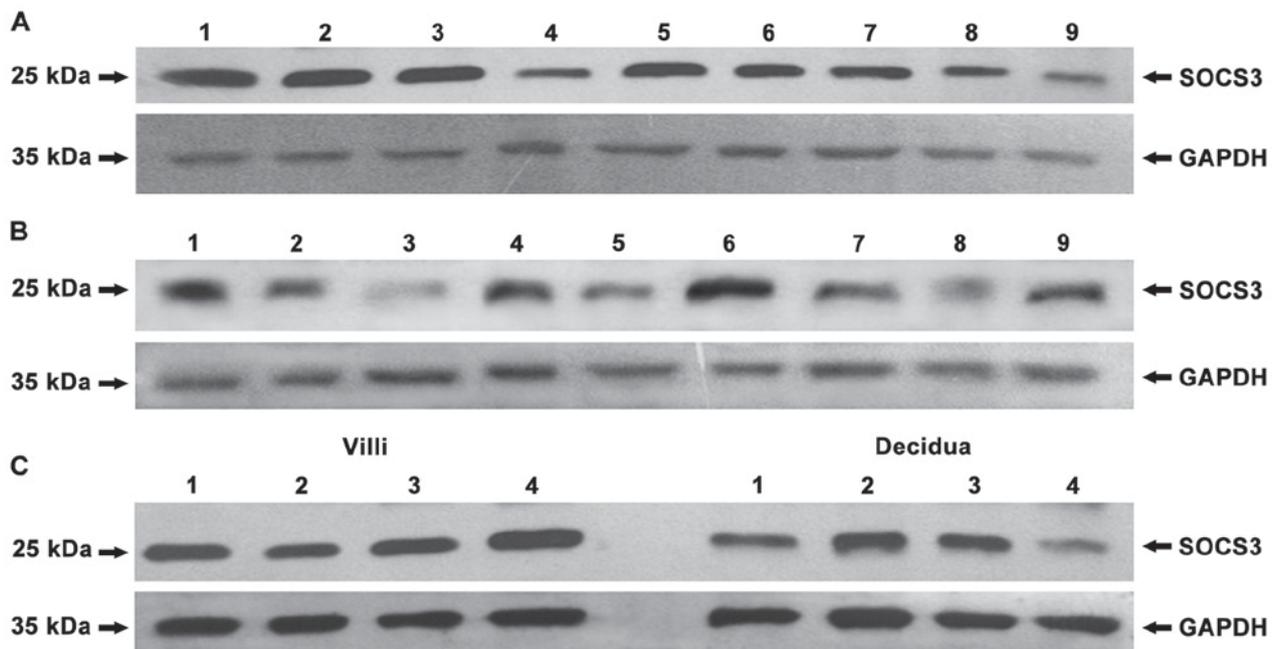


Figure 3. (A) Expression of SOCS3 in chorionic villi and (B) decidua. (C) Comparison of the difference in expression of SOCS3 between chorionic villi and decidua. Labels 1-9 represent different samples of the patients. Data are representative of three independent experiments. SOCS-3, suppressors of cytokine signaling; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

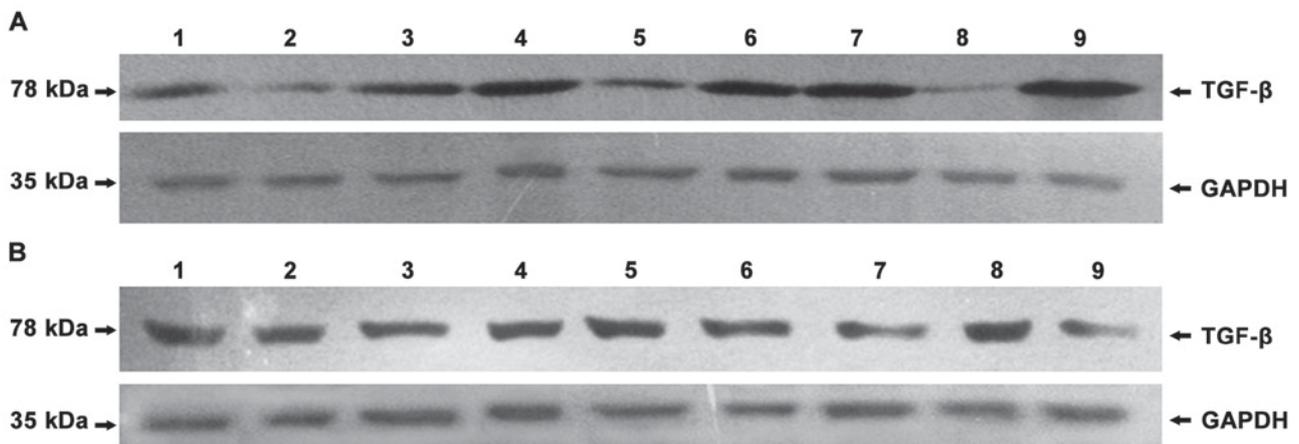


Figure 4. (A) Expression of TGF- β in chorionic villi and (B) decidua. Labels 1-9 represent different samples in each group. Data are representative of three independent experiments. TGF- β transforming growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

the tryptophan metabolite kynurenine is subsequently involved in the inhibition of T and NK cells, inducing the generation of regulatory T (Treg) cells, changing the immune microenvironment *in vivo* and directly regulating the immune response (7,8). Our results indicate that the increased expression of IDO in chorionic villi and decidua may downregulate the immune response mediated by local T-cells, which could prevent the rejection of the fetus by the maternal immune response. Therefore, the factors that regulate IDO protein levels in terms of mediating maternal-fetal tolerance at this site are worthy of further investigation. Moreover, Hönig *et al* (9) and Kudo *et al* (10) have reported that IDO is expressed in the chorionic villi and decidua of normal human pregnancies, while IDO protein and mRNA expression in chorionic villi and decidua have been detected by immunohistochemical and PCR methods (11). Although different technical methods

were used, the results of our study are consistent with these previous studies. Increasing evidence suggests that IDO is expressed in endothelial cells and in syncytiotrophoblasts of early or late placental tissues and that IDO is highly expressed in invasive extravillous trophoblast cells in close contact with the maternal immune system (9). Here, we have used western blot analysis and immunohistochemical methods to confirm that the expression of IDO in human decidua is stronger than that in human chorionic villi, which is in keeping with the above previous studies.

Our results show that SOCS3 is present in chorionic villi and decidua and that there is no significant difference in the expression of this protein between the two tissues. Previous studies have indicated that SOCS3 is involved in trophoblast differentiation and the formation of the placenta, that SOCS3-deficient placentas have decreased spongiotrophoblasts and increased

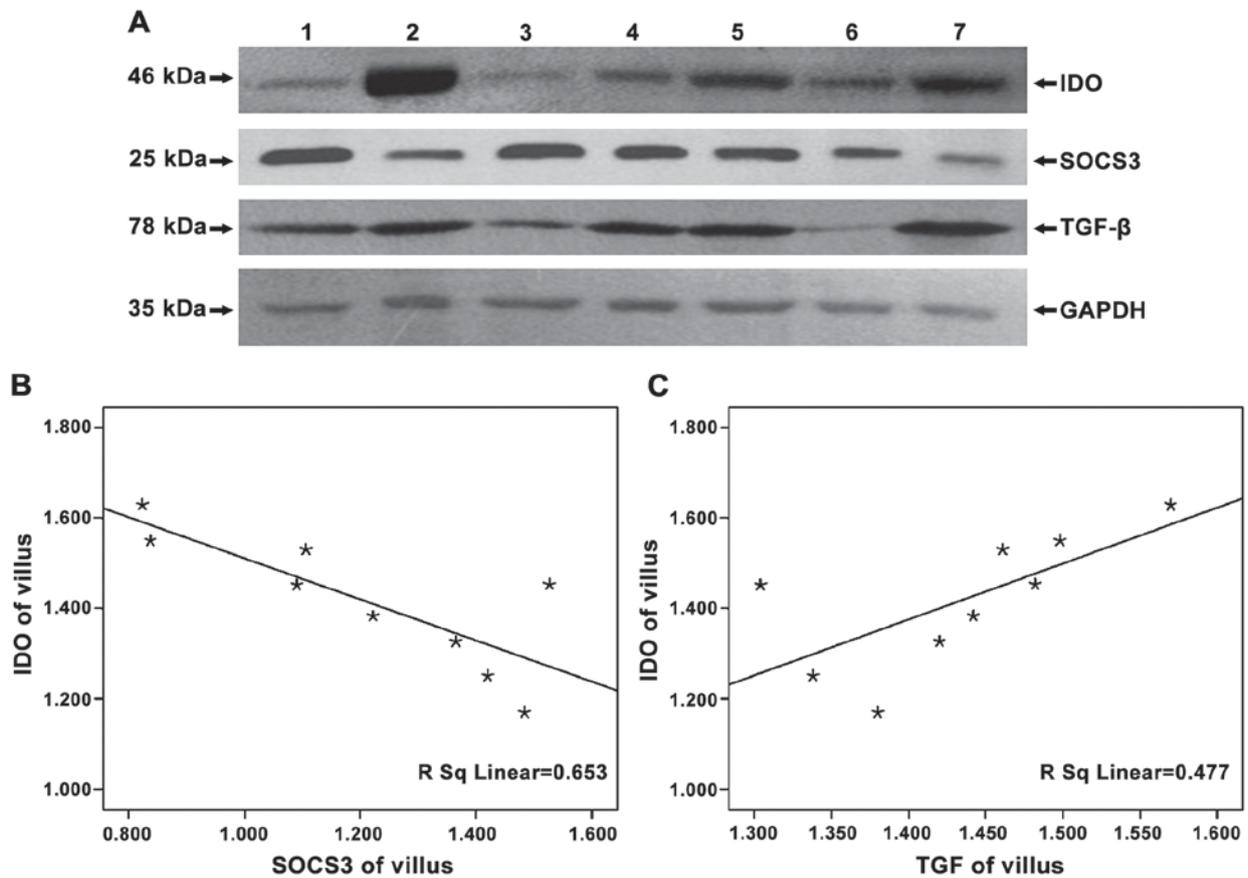


Figure 5. (A) Expression of IDO, SOCS3 and TGF-β in chorionic villi. Scatter diagrams of the correlation of IDO expression with (B) SOCS3 and (C) TGF-β in chorionic villi, respectively. Expression of IDO is negatively correlated with SOCS3, and positively correlated with TGF-β. Data are representative of three independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IDO, indoleamine 2,3-dioxygenase; SOCS-3, suppressors of cytokine signaling; TGF-β transforming growth factor.

trophoblast secondary giant cells and that SOCS3 negatively regulates trophoblast giant cell differentiation. In addition, SOCS3 regulates the development of the placenta by negatively affecting receptor signaling of leukemia inhibitory factor (2,12).

Studies have demonstrated that SOCS3 is able to downregulate the expression of IDO in mouse unfractionated dendritic cells transfected with IDO. The reason for the downregulation may be that the IDO molecule contains two immunoreceptor tyrosine-based inhibitory motifs (13,14) with different molecular chaperone binding, either to promote the degradation of IDO, or to activate its signaling activity and maintain the original enzyme activity (15). If SOCS3 anchors in the phosphorylated ITIM of IDO, IDO is bound to the E3 ubiquitin enzyme complex and degraded (16,17). If the ITIM on IDO molecules combines with tyrosine phosphatase, the expression of IDO protein is enhanced. Our previous study demonstrates that the negative correlation of expression between SOCS3 and IDO exists in the chorionic villi and decidua of women in early pregnancy, and reveals the possibility that SOCS3 may participate in immune regulation via the degradation of IDO at the maternal-fetal interface. It is tempting to speculate that the degradation of IDO may be a new function of SOCS3 in human chorionic villi and decidua. However, whether or not SOCS3 can degrade IDO at the maternal-fetal interface requires further study. Although

the expression of SOCS3 and IDO is negatively correlated, the expression of SOCS3 and IDO coexist. To some extent, IDO remains stably expressed in human chorionic villi and decidua, suggesting that other factors may be involved in regulating and maintaining stable expression of IDO.

TGF-β transmits signals via the Smad and mitogen-activated protein kinase signaling pathways, which regulate the proliferation, differentiation and invasion of trophoblast cells (18) and promote the process of endometrial decidualization. In this study, western blot analysis and immunohistochemistry were used to detect the expression of TGF-β protein in human chorionic villi and decidua of early gestation and the results were consistent with that of previous studies (19). Studies have shown that TGF-β induces immunotolerance by inhibiting B lymphocytes (20), downregulating major histocompatibility complex antigen expression in target cells (5,21) and amplifying T-cells *in vivo* and *in vitro*. Fallarino *et al* and Pallotta *et al* (6,22) found that TGF-β could upregulate IDO expression *in vitro* cultured cells. IDO gene silencing technology can abolish increased expression of IDO by TGF-β. In contrast, 1-methyl tryptophan, as an IDO inhibitor, cannot inhibit the upregulation of induced TGF-β. In 2013, Hanks *et al* (23) also demonstrated that the expression of IDO was upregulated by TGF-β in the tumor microenvironment in animals. Further studies have found that TGF-β is able to phosphorylate the ITIM of IDO and induce its upregulation.

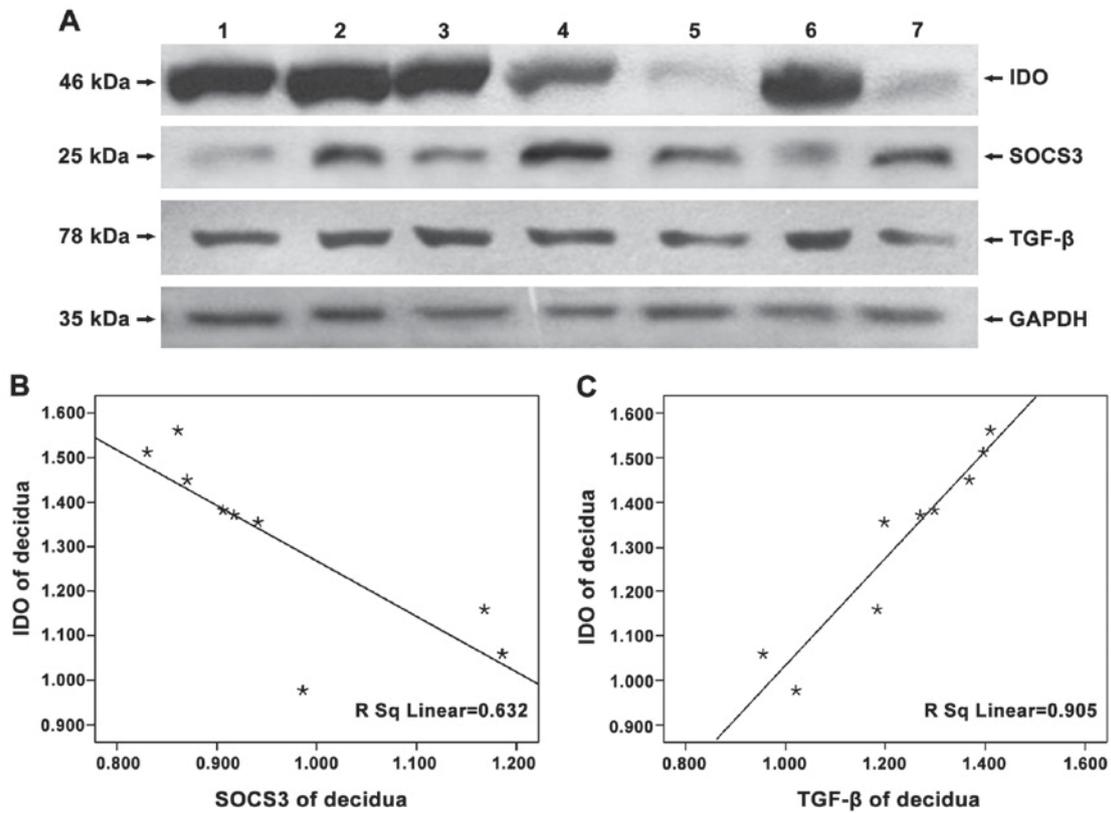


Figure 6. (A) Expression of IDO, SOCS3 and TGF- β in decidua. Scatter diagrams of the correlation of IDO expression with (B) SOCS3 and (C) TGF- β in decidua, respectively. The expression of IDO is negatively correlated with SOCS3, and positively correlated with TGF- β . Data are representative of three independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. IDO, indoleamine 2,3-dioxygenase, SOCS-3, suppressors of cytokine signaling, TGF- β transforming growth factor.

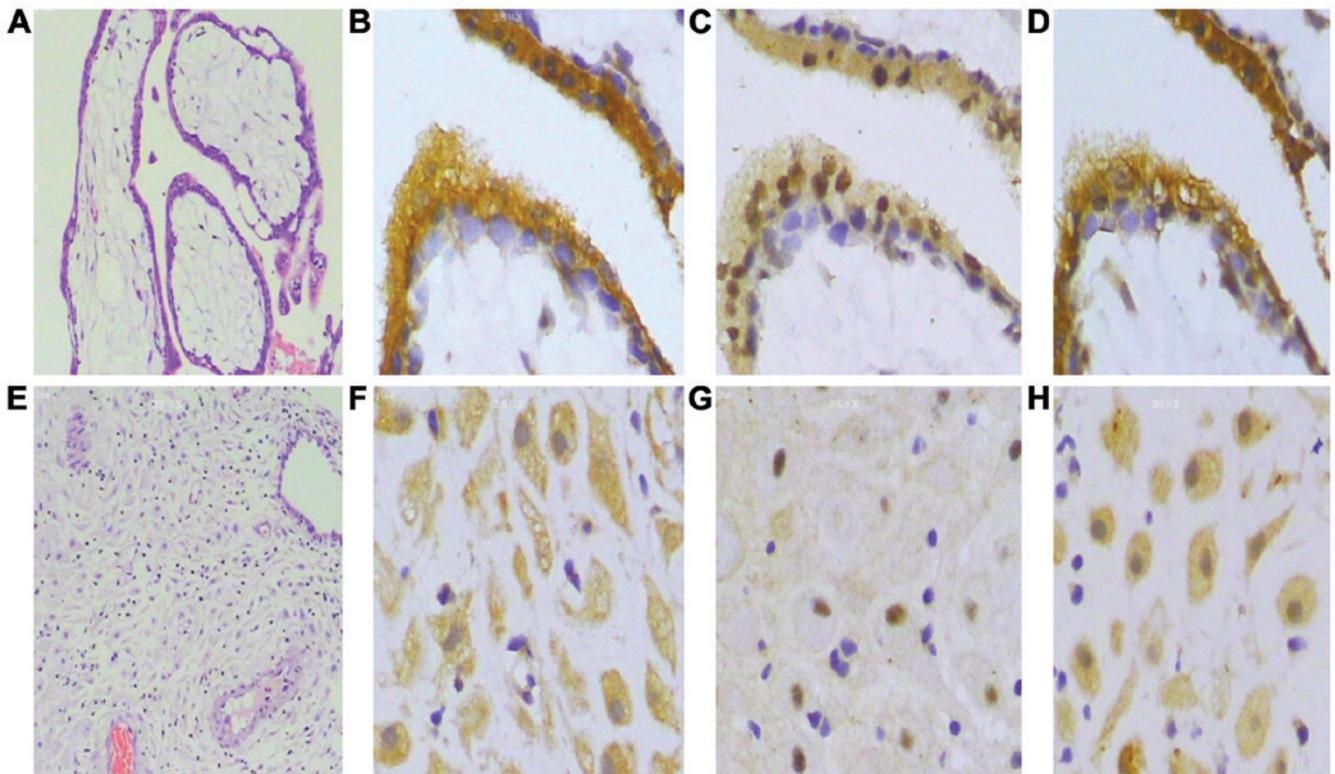


Figure 7. Photomicrograph of (A) chorionic villi and (B) decidua after hematoxylin and eosin (H&E) staining (x100 magnification). Staining for IDO, SOCS3 and TGF- β revealed their presence in the chorionic villi (B-D) and decidua (E-G), respectively (x400 magnification). Images were taken from the same chorionic villi (A-D) and (E-H) decidua sample, respectively. Staining of human first trimester chorionic villi was localized to cytotrophoblasts (B-D). IDO (B-F) and TGF- β (D-H) were present in the cytoplasm, but SOCS3 was present in both the cytoplasm and nucleus (C-G).

Table I. Immunohistochemical staining for IDO and SOCS3 in chorionic villi and decidua from normal pregnancies.

X (intensity of SOCS3 immunostaining)	Y (Intensity of IDO immunostaining)				Total
	+	++	+++	++++	
+	2	5	5	1	13
++	5	6	0	0	11
Total	7	11	5	1	24
Linear-By-linear association	6.031				
Asymp. Sig. (2-sided)	0.014				
Spearman's correlation	-0.511				
Approx. Sig.	0.011				

The results from linear-by-linear association are 6.031, which shows that there is a linear trend between IDO and SOCS3. The correlation coefficient calculated by Spearman's correlation is -0.511 (P=0.011), further explaining that they are negatively correlated.

Table II. Immunohistochemical staining for IDO and TGF-β in chorionic villi and decidua from normal pregnancies.

X (intensity of TGF-β immunostaining)	Y(Intensity of IDO immunostaining)			Total
	+	++	+++	
-	2	0	0	2
+	1	5	0	6
++	2	10	2	14
+++	0	1	1	2
Total	5	16	3	24
Linear-By-linear association	6.264			
Asymp.Sig (2-sided)	0.012			
Spearman's correlation	0.474			
Approx. Sig.	0.019			

The results from linear-by-linear association are 6.264, which shows that there is a linear trend between IDO and TGF-β. The correlation coefficient calculated by Spearman's correlation is 0.474 (P=0.019), further explaining that they are negatively correlated.

Our results show that the expression of IDO concomitantly increases as the expression of TGF-β increases and that the expression of TGF-β and IDO is positively correlated, hinting that TGF-β may be involved in maintaining immune tolerance by upregulating IDO expression at the maternal-fetal interface.

In conclusion, our study demonstrates that IDO, SOCS3 and TGF-β are expressed in chorionic villi and decidua, where IDO expression is negatively correlated with SOCS3 and positively correlated with TGF-β. This suggests a possible role for SOCS3 and TGF-β in maintaining immunotolerance by regulating IDO expression at the maternal-fetal interface. Further investigation is needed to elucidate the mechanisms behind the regulation of IDO expression by SOCS3 and TGF-β.

Acknowledgements

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