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Original Article

Effect of regulatory T cells on reducing the placental inflammatory response

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Abstract: To evaluate the effects of regulatory T cells (Tregs) on the placental inflammatory response in a lipopolysaccharide (LPS)-induced preterm birth mouse model. An LPS-induced preterm birth mouse model was established. Tregs were isolated from pregnant mice and injected into different pregnant mice before LPS treatment. The expression levels of fork head family transcription factor (Foxp3), leukemia inhibitory factor (LIF), heme oxygenase-1 (HO-1), transforming growth factor β1 (TGF-β1), and interleukin-6 (IL-6) in the placenta were examined by western blot and real-time reverse transcription polymerase chain reaction. The expression of Foxp3, HO-1, LIF, and TGF-β1 at both the mRNA and protein levels in the placentas from LPS-treated mice was significantly decreased compared with the controls, while the adoptive transfer of Tregs significantly abrogated the changes in the expression of the above factors after LPS treatment. Interestingly, the expression of IL-6 in the placentas was significantly increased after LPS treatment, and this effect was blocked by the adoptive transfer of Tregs. Maternal LPS exposure significantly induced preterm birth and affected the expression of Foxp3, HO-1, LIF, TGF-β1 and IL-6 in placental tissue. Moreover, the adoptive transfer of Tregs completely abrogated the changes in the expression of the above factors after LPS treatment.

Keywords: Foxp3, LPS, preterm birth, placenta

Introduction

Many factors including exposure to environmental triggers, maternal stress, fetal or maternal genetic abnormalities, and hormonal imbalance can lead to preterm delivery. However, intrauterine infection is one of the most common causes for preterm birth due to the activation of inflammatory responses in both maternal and fetal tissues [1]. It has been well-documented that the placentas from preterm deliveries show pathological signs of chorioamnionitis, which can result from a number of different pathogenic agents [2]. The bacteria in placental tissues can stimulate the production of cytokines such as interleukin (IL)-6, IL-8, and tumor necrosis factor-α (TNF-α) from the amniotic epithelium, decidua, and trophoblasts. These cytokines then stimulate the production of prostaglandin and matrix metalloproteinases, which enhance myometrial contractility and weaken the collagen structure of the membranes, thus causing preterm birth [3].

Regulatory T cells (Tregs), a subset of $CD4^+$ T cells, are known to have important functions in regulating immune T cells and suppressing inflammatory responses in a variety of physiological and pathological settings [4, 5]. Tregs have been demonstrated to play critical roles in the establishment and maintenance of active immune tolerance toward the fetus during pregnancy [6]. Intrauterine growth restriction and preterm birth are correlated with fewer circulating Tregs [7]. Although previous studies have demonstrated that the number of Tregs is decreased in the decidua and peripheral blood of spontaneous abortion cases [8, 9], the precise mechanisms for how Tregs provide fetal protection remain elusive. It has been demonstrated that Tregs at the maternal-fetal interface prevent fetal allo-rejection through upregulating the expression of heme oxygenase-1 (HO-1), transforming growth factor β1 (TGF-β1), IL-10, and cytotoxic T-lymphocyte-associated protein 4 [10]. Moreover, Zenclussen *et al*. have

demonstrated that the adoptive transfer of pregnancy-induced $CD4^+CD25^+$ Tregs prevents fetal rejection in a murine abortion model [11, 12]. However, few studies have examined the efficacy of Tregs in intrauterine infection-triggered preterm birth. Furthermore, the effects of the adoptive transfer of Tregs on lipopolysaccharide (LPS)-induced preterm birth and the inflammatory response in the placenta have never been investigated.

Therefore, the aim of the present study was to investigate the effects of the adoptive transfer of Tregs on day 17 of pregnancy on LPS-induced preterm birth and the inflammatory response in the placenta.

Materials and methods

Animals

Ten-week-old BALB/c female mice were pair-mated with C57 male mice of the same age from the Laboratory Animal Center at The First Affiliated Hospital, Xi'an Jiaotong University. The day of vaginal plug appearance was designated as day 0 of gestation. All mice were housed at the mouse facilities in our university, and all animal procedures were approved by the Institutional Animal Care and Use Committee and Ethics Committee of our university.

Treg isolation and generation of the preterm birth mouse model

$CD4^+CD25^+$ fork head family transcription factor 3 $(Foxp3)^+$ Tregs were isolated from the spleen of a healthy pregnant mouse on day 14 by using a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), an LD, two MS columns, and magnetic beads, following the manufacturer's instructions (MACS, Miltenyi Biotech, Germany). Cells were stained with CD4-FITC (Miltenyi). Cell debris and dead cells were excluded from analysis based on the scatter signals and propidium iodide fluorescence. The purity of the Tregs was between 90% and 96% in all experiments. Tregs were counted, diluted to 2×10^5 in 200 μL of PBS, and injected intravenously (IV) into pregnant mice.

Pregnant mice were randomly assigned to four groups: control group, intraperitoneal (IP) injection of 200 μL of phosphate-buffered saline (PBS); LPS group, IP injection of LPS; LPS+PBS group, IP injection of LPS+IV injection of PBS; and LPS+Tregs group, IP injection of LPS+IV injection of Tregs. The mouse model of preterm birth was established as reported previously [13]. For LPS and PBS injection, BALB/c female mice were injected IP with LPS (*Escherichia coli,* serotype 055:B5; Sigma L-2880, dissolved in saline solution) at a dose of 50 μg/kg or the same volume of PBS, at 2 pm and 5 pm on day 17 of pregnancy. For IV injection, 200 μL of the Treg suspension (2×10^5 cells) or PBS was injected at 1 h before the first LPS injection. For sampling, the mice were anaesthetized with 10% chloral hydrate (350 mg/kg), and the placentas from six mice were harvested at 1, 6, and 12 h after the second LPS injection and at delivery and stored at -80°C for the analysis of gene and protein expression.

Real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from placental tissue using a Tissue RNA Prep kit (OMEGA BIO-TEK, INC., Norcross, GA). Reverse transcription was carried out by using PrimeScript™ RT Master Mix (TaKaRa, Shiga, Japan), following the manufacturer's instructions. qRT-PCR was performed on an ABI PRISM 7900 real-time PCR system (Applied Biosystems, Foster City, CA) to evaluate the mRNA levels of HO-1, LIF, Foxp3, IL-6, TGF-β1, and the reference gene β-actin by using SYBR R Premix Ex Taq™ (TaKaRa). The specific primers for HO-1, LIF, Foxp3, IL-6, and TGF-β1 are listed in **Table 1**. The cycle threshold (Ct) indicated the fractional cycle number at which the PCR product was first detected above a fixed threshold. The relative mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method.

Western blot

The placental tissues were lysed in RIPA buffer (Heart Biological, Xi'an, China). The protein concentration was measured using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Total proteins (50 μg for HO-1, LIF, and Foxp3 or 100 μg for IL-6 and TGF-β1) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane. The membrane was preincubated with 5% nonfat dry milk in Tris-buffered saline

Table 1. Primers used for real-time polymerase chain reaction

Gene	Sequence (5'-3')	PCR product size
FOXP3	Forward primer 5'-AGGGCCAGCATAGGTGCAAG-3'	86 bp
	Reverse primer 5'-AGTGCCTGTGTCCTCAATGGTC-3'	
HO-1	Forward primer 5'-CTGGAGATGACACCTGAGGTCAA-3'	150 bp
	Reverse primer 5'-CTGACGAAGTGACGCCATCTG-3'	
LIF	Forward primer 5'-AAGAATCAACTGGCACAGCTCAA-3'	191 bp
	Reverse primer 5'-AGGTATGCGACCATCCGATACA-3'	
IL-6	Forward primer 5'-CAACGATGATGCACTTGCAGA-3'	142 bp
	Reverse primer 5'-CTCCAGGTAGCTATGGTACTCCAGA-3'	
TGF-β1	Forward primer 5'-TACGGCAGTGGCTGAACCAA-3'	154 bp
	Reverse primer 5'-CGGTTCATGTCATGGATGGTG-3'	
Actin	Forward primer 5'-CATCCGTAAAGACCTCTATGCCAA-3'	171 bp
	Reverse primer 5'-ATGGAGCCACCGATCCACA-3'	

Table 2. Antibodies used for western blot

Antibody	Host Species	Dilution	Specificity	Manufacturer
FoxP3	Mouse	1:1000	Placental tissues	CST
LIF	Mouse	1:100	Placental tissues	SNATA
HO-1	Mouse	1:200	Placental tissues	SNATA
TGF-β1	Mouse	1:200	Placental tissues	SNATA
IL-6	Mouse	1:200	Placental tissues	SNATA

Table 3. Effect of Tregs on LPS-induced preterm delivery in mice

	Number of mice delivered					Rate of preterm delivery	Delivey day (mean)
	Preterm			Term			
	n	Day 17	Day 18	Day 19	Day 20		
Control	6	0	0	1	5	0%	19.8
LPS	6	0	6	0	0	100%	18.0
LPS+PBS	6	0	6	0	0	100%	18.0
LPS+Tregs	6	0	5	1	0	83%	18.1

(TBS) for 1 h and incubated with different primary antibodies (**Table 2**) overnight at 4°C. After three washes in TBS, the membrane was incubated with goat anti-rabbit or rabbit anti-goat secondary antibody for 1 h at room temperature. After three washes in TBS containing Tween-20, positive bands were detected by Immobilon™ Western chemiluminescence HRP Substrate (EMD Millipore, Billerica, MA) using MR Imaging film (Bio-Rad).

Statistical analysis

All data are expressed as mean ± standard deviation. Data were analyzed using two-way analysis of variance, and IBM SPSS version 21 software was used for statistical analysis. A value of $P < 0.05$ was considered to be statistically significant.

Results

Adoptive transfer of Tregs does not affect LPS-induced preterm birth

In this study, we isolated Tregs from the spleen of healthy BALB/c female mice on day 14 of pregnancy, and 2×10^5 Tregs were injected IV into different healthy BALB/c female mice on day 17 of pregnancy at 1 h before LPS injection. Neither maternal death nor fetal death *in utero* was observed in any of the four groups. Compared with the control group, the LPS treatment group (two 50 μg/kg LPS injections at a 3-h interval) showed 100% preterm delivery (**Table 3**). However, IV injection of both Tregs and PBS did not affect preterm delivery in the LPS-induced preterm mouse models (**Table 3**).

Effects of Treg injection on the expression of FOXP3 in placental tissue

As Foxp3 is a master marker for Tregs [14], we first compared the expression level of Foxp3 in the placental tissues of the different groups. Compared with the control group, the expression of Foxp3 mRNA and protein in the placentas from the LPS group was significantly decreased at 1, 6, and 12 h after the second LPS injection ($P < 0.001$, **Figure 1**). Moreover, the adoptive transfer of Tregs significantly increased Foxp3 mRNA and protein expression in the placentas from the LPS+Treg group at 1, 6, and 12 h, compared with the LPS and LPS+PBS groups ($P < 0.001$, **Figure 1**). Furthermore, there was no significant difference in Foxp3 mRNA or protein expression at any of the time points between the control and LPS+Treg groups ($P > 0.05$, **Figure 1**). However, the expression of Foxp3 mRNA and protein was

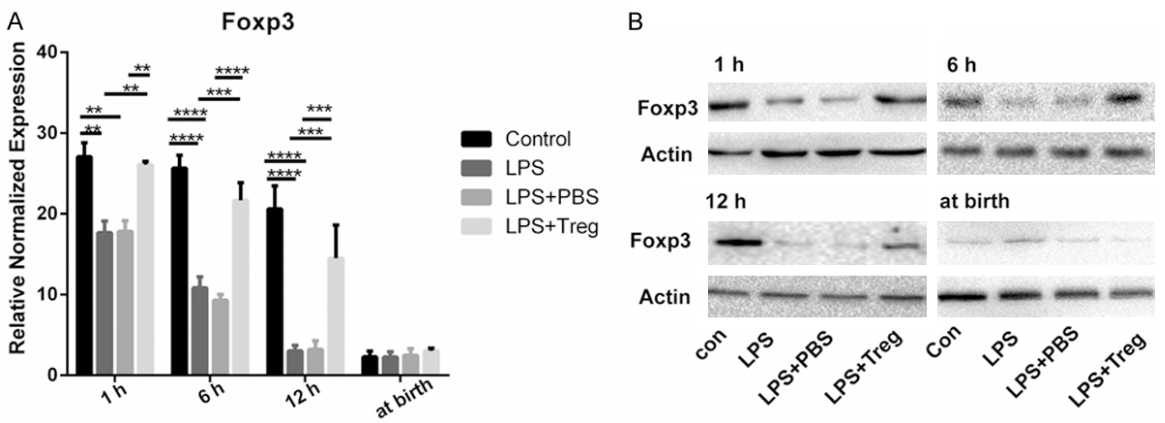

Figure 1. Expression of FOXP3 mRNA and protein in placental tissues after different treatments. A. Real-time PCR analysis of FOXP3 mRNA expression in the placenta in the four groups. Data were analyzed using two-way analysis of variance (n = 6), **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. B. Western blot analysis of FOXP3 protein expression in the placenta in the four groups.

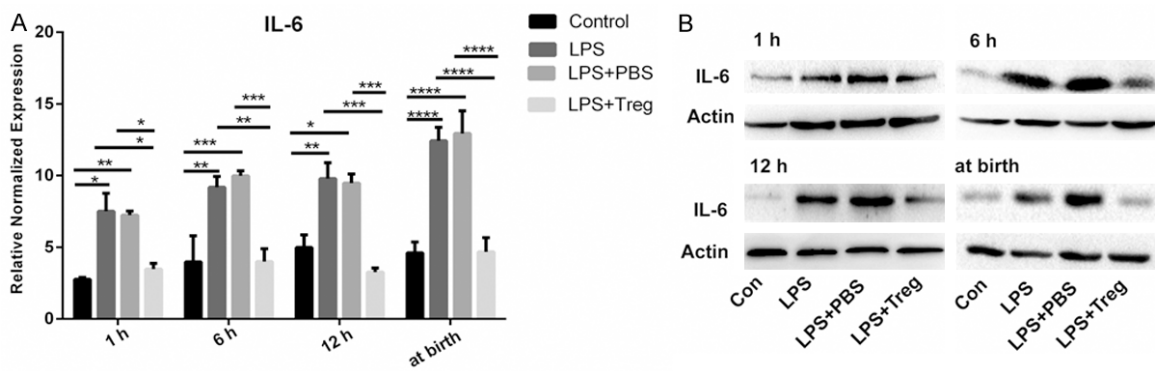

Figure 2. Expression of IL-6 in placental tissues after different treatments. A. Detection of IL-6 mRNA expression in the placenta in the four groups. Data were analyzed using two-way analysis of variance (n = 6), **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. B. Western blot analysis of IL-6 protein expression in the placenta in the four groups.

at the lowest level at birth, and there was no significant difference among the four groups (P > 0.05, **Figure 1**).

Effect of Treg injection on the expression of IL-6 in placental tissue

Intrauterine infection activates the innate immune system and stimulates the release of proinflammatory cytokines such as IL-6 in the placenta [15]. To evaluate the effects of Tregs on the inflammatory response in the placenta, we measured the mRNA and protein expression of IL-6 in the placenta at 1, 6, and 12 h after the second LPS injection and at birth. Maternal LPS administration (LPS and LPS+PBS groups) significantly increased the mRNA and protein expression of IL-6 in the placenta at all examined time points, compared with the control group (P < 0.01, **Figure 2**); while the adoptive transfer of Tregs significantly blocked LPS-induced upregulation of IL-6 expression at both the mRNA and protein levels (LPS+Treg group vs. LPS group, P < 0.05 or P < 0.01, **Figure 2**). Additionally, there was no significant difference in IL-6 mRNA or protein expression at any time points between the control and LPS+Treg groups (P > 0.05, **Figure 2**).

Effect of Treg injection on the expression of HO-1, LIF, and TGF-β1 in placental tissue

It has been demonstrated that the mRNA levels of LIF, TGF-β1 and HO-1 at the fetal-maternal interface are dramatically upregulated after Treg transfer [16]. Here, we observed that the mRNA expression of HO-1, LIF, and TGF-β1 in the placentas from the LPS group was signifi-

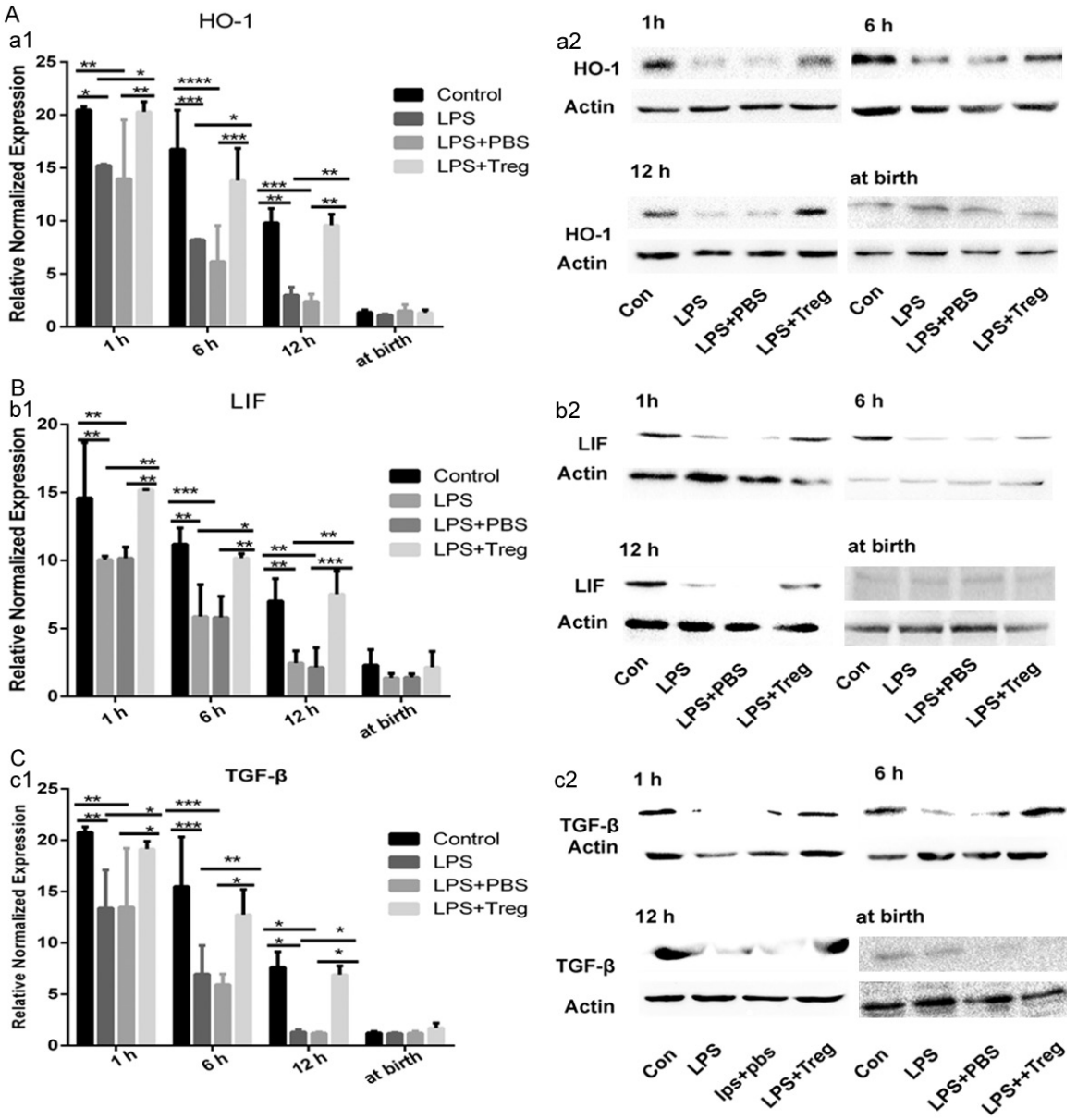

Figure 3. Expression of HO-1, LIF, and TGF-β1 in placental tissue after different treatments. a1-c1. mRNA expression of HO-1, LIF, and TGF-β1 in the placenta was examined by qRT-PCR, n = 6, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. a2-c2. Protein expression of HO-1, LIF, and TGF-β1 in the placenta by western blot.

cantly decreased at 1, 6, and 12 h after the second LPS injection, compared with the control group (P < 0.001, **Figure 3a1-c1**). However, the mRNA expression of HO-1, LIF, and TGF-β1 in the LPS+Treg group at 1, 6, and 12 h after the second LPS injection was significantly increased compared with the LPS and LPS+PBS groups (P < 0.05, **Figure 3a1-c1**). There was no significant difference in the mRNA expression of HO-1, LIF, or TGF-β1 at birth among the four groups (P > 0.05, **Figure 3a1-c1**). Moreover, no significant difference in the mRNA expression of HO-1, LIF, or TGF-β1 was observed at any time points between the control and LPS+Treg groups. Furthermore, the protein expression patterns of HO-1, LIF, and TGF-β1 at all time points were completely consistent with the mRNA expression patterns among the four groups (**Figure 3a2-c2**).

Discussion

It has been demonstrated previously that the number of Tregs is higher in allogeneic preg-

nant mice than in syngeneic pregnant mice [17]. Hybrid pregnant mice have been shown to have 100% preterm deliveries when they were treated with 50 μg/kg LPS twice at a 3-h interval on day 17 of pregnancy [13]. Therefore, in this study, we used murine BALB/c × C57 pregnant mice as a preterm delivery model and observed 100% preterm delivery after LPS treatment.

A few studies have focused on the relationship between LPS and Tregs in different diseases [18, 19]. However, the effect of the adoptive transfer of Tregs in LPS-induced preterm delivery has been rarely studied. Our data demonstrated that Treg injection did not affect the incidence of preterm birth in the LPS-induced preterm delivery mouse model, which is not consistent with our hypothesis that Tregs may reduce the incidence of preterm birth after LPS treatment. It is well accepted that the origin, expansion, migration, and function of Tregs during pregnancy are complex [20-22]. Immune cells such as T cells, B cells, dendritic cells, and macrophages are activated at the fetal-maternal interface during pregnancy and produce chemokines; therefore, they are involved in Treg attraction and regulation. Tregs are essential mediators of maternal immune adaptation during embryo implantation [12, 23], but the adoptive transfer of Tregs after day 4-5 of pregnancy did not inhibit early abortion. Additionally, intrauterine infection-induced preterm delivery results from extremely complex inflammatory mechanisms. Thus, it is not sufficient to evaluate the effects of Tregs on LPS-induced preterm delivery solely based on the incidence of preterm delivery.

IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory activities [24]. It is one of the key inflammatory cytokines that is upregulated in various inflammatory conditions [25]. As a proinflammatory cytokine, increased expression of IL-6 in amniotic fluid is closely associated with brain damage in premature birth [15, 26]. Conversely, as an anti-inflammatory cytokine, IL-6 plays a crucial role in the inhibition of autoimmune tissue inflammation [25]. Here, we found that Treg injection significantly blocked LPS-induced upregulation of IL-6 expression at both the mRNA and protein levels in placental tissue. These results suggest that Treg therapy may reduce the LPS-induced inflammatory response in the placenta by inhibiting IL-6 expression, but this speculation needs further investigation.

TGF-β1, a bipolar cytokine that can both trigger and inhibit the immune system in neonatal sepsis [27], has been shown to be one of the molecules involved in Treg functions *in vitro*. Many studies have demonstrated that LIF plays a primary role in embryo implantation, both in humans and mice [28, 29]. Blitek *et al*. [30] have reported that LIF is one of the important components of embryo-uterine interactions during early pregnancy in the pig and may contribute to successful conceptus implantation. HO-1 is the only inducible HO isoform and breaks down heme into carbon monoxide (CO), biliverdin, and free iron. CO and bilirubin (a downstream product of biliverdin processing) account for the anti-inflammatory, antiapoptotic, antioxidant, and cytoprotective properties of HO-1 [31, 32]. Zenclussen *et al*. [33] observed that HO-1 was significantly downregulated in placental tissues from allo-pregnant mice undergoing abortion and that HO-1 expression was associated with early embryonic death [12]. HO-1 downregulation has a high potential to be harmful at the maternal-fetal interface, since a large amount of free heme will easily incorporate into endothelial cells and cause oxidative injury. A low level of CO can expand the inflammatory response by promoting the expression of proinflammatory genes and inhibiting the production of anti-inflammatory cytokines [34]. It has been demonstrated that the expression of HO-1, LIF, and TGF-β1 at the fetal-maternal interface of the murine abortion-prone model is mediated by the adoptive transfer of Tregs [10, 16]. Our data also showed that the expression of TGF-β1, LIF, and HO-1 in the placenta was significantly decreased in LPS-induced preterm mice, while their expression was completely reversed after the adoptive transfer of Tregs to LPS-induced preterm birth mice. Our data indicate that LPS treatment in pregnant mice can trigger the inflammatory response in the placenta, while the adoptive transfer of Tregs can completely suppress the inflammatory responses induced by LPS.

In conclusion, our data demonstrate that the adoptive transfer of Tregs can suppress the inflammatory response in the placenta of LPS-induced preterm birth mice, which is achieved by mediating the expression of IL-6, HO-1, LIF, and TGF-β1 in the placenta.

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Disclosure of conflict of interest

None.

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