Prolactin modifies the in vitro LPS-induced chemotactic capabilities in human fetal membranes at the term of gestation

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Abstract

Problem: Immune responses of fetal membranes involve the production of chemotactic mediators causing infiltration of maternal and fetal leukocytes, intrauterine inflammation and potentially the disruption of maternal-fetal tolerance. Prolactin (PRL) has deep immunoregulatory effects in the fetal-maternal interface. We aimed to test the in vitro PRL effect upon chemotactic capacities of human fetal membranes.

Method of Study: Fetal membranes and umbilical cord blood were collected from healthy non-laboring caesarean deliveries at term. Fetal membranes were cultured in Transwell® frames to mimic the barrier function between chorionic and amniotic sides. Tissues were treated with PRL, Lipopolysaccharide (LPS), or both simultaneously. Then, RANTES, MCP-1, MIP-1α, IP-10, and PECAM-1 were quantified in a conditioned medium by chorion-decidua or amniotic sides. The chemotaxis of subsets of migrating mononuclear cells from umbilical cord blood was evaluated in a Boyden Chamber in response to the conditioned medium by both sides.

Results: Lipopolysaccharide stimulates the production of RANTES, MCP-1, MIP-1α, and PECAM-1 in chorionic decidua, while MIP-1α and PECAM-1 only increase in amnion. PRL decrease RANTES, MCP-1, and MIP-1 only in chorionic decidua, but PECAM-1 was decreased mainly in amnion. The leukocyte migration was regulated significantly in response to the conditioned medium by the amnion, increase in the conditioned medium after LPS treatment, contrary with, the leukocyte migration decreased in a significant manner in response to conditioned medium after PRL and LPS-PRL co-treatment. Finally, T cells were the most responsive subset of cells.

Conclusions: Prolactin modified in a tissue-specific manner the chemotactic factor and the leukocyte migration differentially in fetal membranes.

Keywords
annim, leukocytes, LPS, migration, prolactin, T cells
1 | INTRODUCTION

Fetal membranes constitute a selective barrier with physical and immunological functions essential for maintaining the fetus in an immunologically privileged milieu of placenta and are composed of two compartments of choriodecidua and amnion. These adjacent structures are non-vascularized, however, infiltrating leukocytes are observed during labor. The onset of labor is led by the secretion of cytokines such as TNF-α, IL-1β, and IL-6 produced by the myometrium, cervix, and fetal membranes. This inflammatory environment causes the activation of the myometrium, cervical dilation, and rupture of fetal membranes in a coordinated manner. Secondary to this initial inflammatory stage, gestational tissues secrete chemotactant such as IL-8, RANTES, MCP-1, and MIP-1α, which recruit leukocytes from maternal and fetal circulation. Progressive accumulation of immune cells in the myometrium, decidua, fetal membranes, and amniotic cavity enhance the inflammatory environment and tissue remodeling to induce labor. Several works have demonstrated that fetal membranes possess immunological capabilities that play an important part in the immune defense against pathogens. However, higher immune response caused by intrauterine infections can trigger a premature onset of labor before the 37 weeks of gestation. In this scenario, inflammatory responses increase the number of immunocompetent cells (neutrophils, monocytes, and lymphocytes) into the fetal membranes, promoting a premature rupture of chorioamniotic membranes. On the other hand, Prolactin (PRL) is a neuroendocrine hormone that was originally described as a product of secretion by the pituitary gland, and involved in the development of breast and milk production. During pregnancy, decidua is the main source of extra-pituitary PRL production, that crosses the fetal membranes and stores in the amniotic cavity, reaching levels of 4000 ng/mL, which are 10 times greater than maternal and fetal serum concentrations. PRL performs important functions at the maternal-fetal interface regulating the osmolarity in fetal membranes, promoting the proliferation and migration of extravillous trophoblast, maintaining higher levels of progesterone and performing immunomodulatory effects. PRL decreases the secretion of inflammatory cytokines such as IL-6 in decidual cells, decreases the expression and secretion of TNF-α and IL-1β induced by Lipopolysaccharide (LPS) from Escherichia coli in the placenta, and decreases the secretion of prostaglandin E₂ from fetal membranes. In our laboratory, we demonstrated that PRL inhibits the secretion of TNF-α, IL-1β and MMP-9, and limits the secretion of pro-inflammatory cytokines induced by LPS in fetal membranes with differential effects between choriodecidual and amniotic sides.

Considering the background, the goal in this work was to evaluate the effect of PRL on the chemoattractant potential of LPS-activated fetal membranes in an in vitro model.

2 | MATERIAL AND METHODS

2.1 Biological samples

This study was approved by the Human Ethics, Biosecurity and Research Committees of the Instituto Nacional de Perinatología "Isidro Espinosa de los Reyes" (212250–3210–21205–01–15) and UMAE Hospital de Ginecología y Obstetricia No. 4 "Luis Castelazo Ayala" (IMSS R-2017–785–013) in Mexico City. All patients accepted to participate in the study by signing a written informed consent form.

Fetal membranes, and cord blood samples from healthy women at term (range 37.5–39.5 week of pregnancy), uncomplicated, and singleton pregnancies were collected after elective cesarean section, and were performed before the onset of labor. No patient was under medical treatment or received medication. Patients with a history of cervicovaginal infection, penicillin allergy, chronic hypertension, polyhydramnios, oligohydramnios, diabetes mellitus, or preeclampsia were excluded from the study. Additionally, microbial analyses of all samples were conducted to preclude the presence of chorioamniotic infection. All sample tissues were examined by macroscopic and routine histopathology techniques to confirm the normal morphology of these tissues and the absence of inflammation signals. The clinical data of participants are summarized in Table 1.

The biological samples were obtained and transported in sterile conditions at birth. Umbilical cord blood was collected in Heparin-containing tubes (BD Vacutainer, Becton Dickinson) by venipuncture of the umbilical vein. Fetal membranes were cut five centimetres above the placenta and transferred to sterile sodium chloride solution 0.9% (PiSA).

2.2 Culture and stimulation of chorioamniotic membranes

All samples were manipulated under sterile conditions. We used the two-compartment tissue culture system, which has been previously standardized by our group. Briefly, we exhaustively rinse the chorioamniotic membranes with sodium chloride solution 0.9% to eliminate the remaining blood clothes; we cut explants of whole membranes that were manually mounted and held with silicone rubber rings to be placed into a Transwell® frame (Costar) with the

| TABLE 1 Demographic and clinical characteristics of the study population |
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| **Clinical data** | **Mean ± SD (Range)** |
| Maternal age (years) | 32.0 ± 5.2 (20–39) |
| Maternal pregestacional weight (kg) | 66.1 ± 8.0 (57–81) |
| Maternal gestacional weight (kg) | 72.6 ± 6.2 (57–81) |
| Gestational age (weeks) | 38.6 ± 1.0 (37–39) |
| Preganacies (number) | 2.1 ± 1.0 (1–3) |
| Newborngender (Female-Male %) | 50–50 |

SD, Standard deviation.
polycarbonate membrane previously removed. Thus, created two independent compartments that allowed us to test the compartmentalized secretion of soluble factors. The choriodecidua were faced to the upper chamber of the Transwell® and the amnios to the lower chamber.

The mounted explants were placed in a 12-well tissue culture plate (Costar). One mL of DMEM with phenol red (Blowest; Nuaille, FRA) and supplemented with 0.2% lacticalbumin hydrolysate, 1 mM sodium pyruvate, 1× antibiotic-antimycotic solution (penicillin 100 U/mL, streptomycin 100 µg/mL and amphotericin B 0.25 µg/mL) (GIBCO, BRL) were added to each side of the chamber and were incubated in 5% CO₂ at 37°C for a day. At the beginning of culture, 4000 ng/mL of PRL (Peprotech) were added to the upper and lower chambers. At the second day, old medium was replaced by 1 mL of fresh culture medium in accord with the experimental treatments that included: (1) culture media; (2) PRL [4000 ng/mL]; (3) LPS [500 ng/mL]; (4) LPS +PRL; additionally, we include an anti-inflammatory control that comprises LPS +Dexamethasone [200 nM] cotreatment and the cotreatment of LPS +Methotrexate [50 µM], a non-specific inhibitor of PRL-receptor signaling. The LPS stimulation was added just on the choriodecidua in all treatments. Samples were incubated 24 h in 5% CO₂ at 37°C and the medium from both compartments were collected, divided into aliquots, and stored at −70°C until assay.

2.3 | Quantification of chemotactic factors

The concentrations of chemokines MCP-1 (900-K31; Peprotech), MIP-1α (900-K35; Peprotech), RANTES (900-K33; Peprotech), IP-10 (900-K39; Peprotech), and the adhesion molecule PECAM-1 (DY806-05; R&D Systems) present in cell culture supernatants from maternal and fetal compartments were determined by Sandwich ELISA according to the manufacturer’s instructions.

2.4 | Isolation of umbilical cord blood mononuclear cells (UCBMC)

Using umbilical cord blood samples from donors previously collected into heparinized tubes were diluted 1:1 with sterile sodium chloride solution 0.9% (PISA®). Then, 25 mL of diluted blood was carefully underlaid in 12 mL of Lymphoprep® (Stemcell Technologies) to isolate the leukocytes by density gradient, then centrifuged at 400 g for 40 min at room temperature with the brake turned off. Following the procedure, the band of leukocytes (which represent UCBMC) was recovered and resuspended in Roswell Park Memorial Institute Medium (RPMI-1640; GIBCO Invitrogen) without any supplementation. Leukocyte viability was assessed by trypan blue exclusion and the leukocyte count was determined using a Hemocytometer. Viability was greater than 95%. Leukocyte suspension was stored at 37°C and used within an hour after isolation.

2.5 | Chemotaxis assay

The chemotaxis assay was performed using a Boyden Chamber, where 350 µL of each conditioned medium coming from amnios and choriodecidua were placed in the lower chamber as a chemotactic factor; immediately 1 × 10⁶ UCBMC in 350 µL RPMI were added on top of the polycarbonate membrane Transwell® insert (8 µm pore size; Costar) that correspond to the upper chamber (Figure 1).

Boyden chambers were incubated from 60 minutes at 37°C in humidified air containing 5% CO₂. Afterwards, Transwell inserts containing the non-migrated leukocytes were removed.

Every experimental condition in chemotaxis assay was performed by triplicate; two replicates were used to cell counting and the other one left for immunophenotyping.

To evaluate the proper functioning of the migration assay and determine whether the Prolactin present in the conditioned media could act as a chemoattractant, internal controls were performed, consisting of the use of supplemented DMEM non conditioned by fetal membranes (negative control), supplemented DMEM +10% Fetal Calf Serum (FCS; positive control), and supplemented DMEM +4000 ng/mL of PRL.

2.6 | Migrant Leukocyte counting

UCBMC that migrated through the filter into the bottom wells were collected, stored, and then stained with the CyQUANT® GR dye kit (Invitrogen) following manufacturer’s instructions and quantified by interpolation from the standard curve using a BioTek® Synergy HTX microplate reader.

2.7 | Immunophenotyping of migrant UCBMC

After we collected and centrifuged the migrated cells to get a pellet, these were stained and analyzed by flow cytometry to identify the phenotype of attracted heterologous leukocytes using conjugated monoclonal antibodies BD Multitest®: total leukocytes/anti-CD45-PerCP (2D1/HLe-1), T cells/anti-CD3-FITC (SK7), B cells/anti-CD19-APC (SJ25C1), NK cells/anti-CD16++PE (B73.1)/anti-CD56-PE (NCAM 16.2; BD Bioscience) and Monocytes/anti-CD14-PE/Cy7 (HCD14; Biologend). The flow cytometer FACSAria III (BD Bioscience) was set to analyze at least 10 000 events, the results were analyzed using the BD FACSDiva 8.0.2 software (BD Bioscience).

2.8 | Statistical analysis

The chemokine immunoassay results were analyzed by non-parametric analysis of variance Kruskal-Wallis. PECAM immunoassay results, the differences between experimental treatments in the
number of total leukocyte migration and immunophenotyping of immune cells were analyzed by one-way analysis of variance (ANOVA) using Sigma Stat v 11 (Systat Software, SigmaStat Version 13, from Systat Software, Inc.; systatsoftware.com). A \( p \)-value \( \leq 0.05 \) was considered statistically significant.

3 | RESULTS

Taking into consideration that in this study, we used biological samples from pregnant women, demographics sensible data of the patients that participate in this study are not publicly available due to privacy or ethical restrictions.

3.1 | Differential secretion profile of chemokines in the conditioned mediums by fetal membranes

After experimental treatments, the concentration of secreted chemokines was measured in supernatants. We observed a differential secretion profile between each compartment in the chorionic decidua culture system. RANTES and IP-10 were mainly detected in the chorionic decidua conditioned mediums, while MCP-1 and MIP-1\( \alpha \) were detected in both chorionic decidua and amniotic conditioned mediums (Figure 2).

In comparison with control, LPS treatment increased significantly the secretion of RANTES (18-fold), MCP-1 (6-fold), MIP-1\( \alpha \) (23-fold), and IP-10 (7.1 fold) in the chorionic decidua region (\( p < 0.001 \)). On the other hand, the same stimulus only significantly increased the secretion of MIP-1\( \alpha \) (8-fold) in the amniotic region compared to control (\( p < 0.001 \)).

As a control, PRL does not induce the production of secreted chemokines in both compartments. Interestingly, the co-treatment with PRL plus LPS, PRL significantly diminish the secretion of MCP-1 and MIP-1\( \alpha \) induced by LPS, in 50% and 70% respectively (\( p < 0.001 \)), compared to only LPS treatment (Figure 2A,B); but the secretion of RANTES and IP-10 was not decreased in response to this co-treatment (Figure 2C,D).

For amnion, none of the chemokines induced by LPS was significantly downregulated by the co-treatment with LPS plus PRL.

The co-treatment with LPS and Dexamethasone was used as an experimental control of anti-inflammation, which decreased the secretion of RANTES, MCP-1, and MIP-1\( \alpha \) induced by LPS in chorionic decidua (\( p < 0.001 \)); and the MIP-1\( \alpha \) secretion induced by LPS in amnion (\( p < 0.001 \)), as expected.

On the contrary, the co-treatment with Methotrexate significantly reversed the inhibitory effect of PRL increasing the secretion of MIP-1\( \alpha \) in chorionic decidua at similar level induced by the LPS stimulus. The MCP-1 and RANTES secretion showed a tendency to increase in response to co-treatment with Methotrexate in chorionic decidua and amnion. These results indicate that chemokine down-regulation is PRL dependent because Methotrexate acts as a pan-JAK inhibitor, blocking the canonical signaling of PRL which depends on the JAK/STAT activation pathway.

3.2 | The differential soluble fraction of PECAM-1 in the conditioned mediums by fetal membranes

In the chorionic decidua compartment, the experimental treatments did not show a significant difference among them. Conversely, in the amniotic compartment, the treatment with LPS increased 1.4-fold of the
PECAM-1 levels in a significant manner in respect to control ($p < 0.001$), the co-treatment with LPS and PRL decreased at 52% the PECAM-1 levels in comparison with the levels induced by LPS treatment ($p < 0.001$).

The PECAM-1 levels in the simple PRL treatment were not differed to control. In the co-treatment with LPS plus Dexamethasone the PECAM-1 levels were significantly minor to LPS levels, even to control levels in both choriodecidual and amniotic compartments ($p < 0.001$). The co-treatment with Methotrexate was not able to revert the PRL effect in comparison to the co-treatment with LPS (Figure 3).

### 3.3 Differential chemotactic capacities of choriodeciduala and amnion conditioned mediums

After the migration assays, where the conditioned mediums by amnion and choriodecidual were used, we counted the migrant cells in the lower chamber.

The internal controls of the chemotaxis assay demonstrate that the supplemented DMEM non-conditioned medium (negative control) and PRL [4000 ng/mL] added to non-conditioned medium does not have chemotactic activity per se in contrast with the positive control (FCS 10%) (Figure 4A).

In experimental control conditions, there are chemotactic factors that increase the migration of leukocytes. A similar pattern in both amnion and choriodecidual conditioned medium was observed for the total migrant cells in response to different experimental treatments, but the statistical differences were only found in amnion (Figure 4B,C).

We observed that the LPS-conditioned amnion medium significantly increased the chemotaxis of total UCBMC in comparison with the experimental control medium ($p < 0.001$), also co-treatment with PRL decreased the leukocyte migration in the conditioned medium amnion ($p < 0.001$). According to the incapacity of PRL to induce secreted chemokines in fetal membranes by itself, the medium coming...
from PRL-exposed tissues did not increase the chemotactic activity, neither choriodecidual nor amniotic conditioned mediums.

Besides, the co-treatment with LPS and Dexamethasone decreased the cell migration in the conditioned medium for both, amnion and choriodecidual, but the media conditioned by the co-treatment with Methotrexate, LPS, and PRL, increased the chemotaxis of UCBMC only in amnion conditioned medium ($p < 0.01$).

### 3.4 Subset of migrant UCBMC

To determine the differential sensitivity of the leukocyte’s subsets in response to the conditioned medium, we analyzed the immunophenotype of chemoattracted UCBMC by flow cytometry. T lymphocytes were the population with the highest migration (Figure 5A), followed by NK cells (Figure 5B); B lymphocytes (Figure 5C) and monocytes (Figure 5D) were found in a smaller proportion. The significant chemotaxis regulation was not observed in B-lymphocytes, NK cells, or monocytes, but in T cells, the pattern of migration was different between treatments.

We found significant changes in the number of chemoattracted T cells, increased 1.3-fold in response to LPS treatment in the amnion conditioned medium ($p < 0.001$) versus experimental control medium. As shown in Figure 5A, in media conditioned by co-treatment with LPS plus PRL or Dexamethasone, T cell chemotaxis decreased by 52% and 62% respectively compared to induced LPS-chemotaxis ($p < 0.001$).

There were no significant changes in the mediums conditioned by choriodecidual treatments (Figure 5A), except in the anti-inflammatory control (co-treatment with LPS and Dexamethasone), in this case, the T cells decreased compared to the chemotaxis observed in the medium conditioned by the LPS treatment and control ($p < 0.001$).
DISCUSSION

Clinical and experimental evidence supports that leukocyte infiltration into the maternal-fetal interface is a critical event during inflammation in term labor and preterm labor with or without infection. This cellular infiltration is regulated by chemokines and cellular adhesion molecules. Previous research demonstrated that fetal membranes have immunological competencies that include the secretion of the chemokine.

Our results demonstrate that under basal conditions, amnion and choriodecidua showed a differential secretion profile of RANTES, MCP-1, MIP-1α and IP-10. However, once stimulation with LPS was done, both regions responded by increasing in a significant manner the differential secretion of all chemokines to their culture medium which supports the function and responsiveness of our ex vivo experimental model. This differential secretion profile agrees with previous works, where fetal membranes are challenged in vitro by bacterial products and virus. These results suggest different capacities of choriodecidua and amnion to recruit a specific subset of immune cells depending on the immunological challenge that is presented. Moreover, in our results, the increased secretion of RANTES, MCP-1, MIP-1α by LPS, were inhibited by PRL as we propose in our hypothesis, in agreement with our previous work, where PRL regulates in a dose-dependent manner the differential secretion of chemokines LPS-induced in chorioamniotic membranes.

RANTES, MCP-1, and MIP-1α are members of CCL chemokines family; and they are the chemokines mostly detected in amniotic fluid during preterm labor with/without infection. RANTES (CCL5) is a potent chemotactic factor to exert the activation of monocytes, T Lymphocytes, involved in the adhesion and migration of T cells into the endothelium. MCP-1 (CCL2) attracts monocytes and macrophages into the inflammatory tissue, where it promotes...
the respiratory burst, lead the macrophage activation and polarization of M1 macrophage, \textsuperscript{31,32} and MIP-1\(\alpha\) (CCL-3) chemoattract neutrophils, basophils, lymphocytes, and monocytes which activate the release of histamine and cytokine production.\textsuperscript{34} The differential in vitro chemokine secretion profile for each region of the fetal membranes observed suggests selective and differential recruitment of immune cells.

To test the hypothesis that each region of fetal membranes influenced by PRL can attract a specific phenotype of immune cells, we used the conditioned media coming from amnion compartment and choriodecidual compartment in Boyden Chamber assays and evaluated the chemotaxis of leukocytes isolation of umbilical cord blood. Surprisingly, contrary with what we expected, only conditioned amnion media regulated differentially the total increased leukocyte migration in response to LPS treatment, and PRL decreased the total number of migrant’s leukocytes.

This effect may be attributed to the downregulation of soluble PECAM-1, which we observed only in amnion conditioned medium and the lower chemokine secretion in this side in comparison to the choriodecidual response. PECAM-1 or CD31 is a transmembrane hemophilic receptor not only highly expressed in endothelial cells but also in granulocytes and leukocytes. The CD31 homophilic interaction between lymphocytes endothelium and neutrophils play a part in the recruitment, extravasation, and transmigration of immune cells into the inflamed tissues.\textsuperscript{35,36} In fetal membranes from spontaneous vaginal term labor, the PECAM-1 and ICAM-1 mRNA expression are upregulated in the decidua, chorion, and amnion regions, indicating the role of the adhesion molecules in the inflammatory processes in these tissues.\textsuperscript{37} Furthermore, the extracellular domain of PECAM-1 is released during tissue damage and endothelial cell apoptosis.\textsuperscript{38} In fact, the higher levels of PECAM-1 in amniotic fluid suggest that the determination of PECAM-1 levels could be used as a marker of intra-amniotic inflammation and the possible development of preterm rupture of membranes.\textsuperscript{39}

These results give an important biological significance to the differential role of each compartment from fetal membranes in their immune response. Unlike amnion, the choriodecidual is in direct contact with a greater number of maternal immune cells throughout the pregnancy. In vitro evidence, has supported that amnion possesses intrinsic immunoregulatory properties, the co-culture with amniotic cells or conditioned media from the amniotic cells inhibits the activation/proliferation of T cells,\textsuperscript{40,41} and the infiltration and survival of neutrophils.\textsuperscript{42,43}

It is known that several hormones can regulate the immune elements and the immunological competences of diverse tissues in the maternal-fetal interface through pregnancy.\textsuperscript{44} Our results suggest that PRL could improve the intrinsic anti-inflammatory properties that have been observed in the amnion, who has a critical location...
in the maternal-fetal interface. We also report that the PRL present in the culture media did not induce the leukocyte migration per se, similar to what Dill and Walker reported\textsuperscript{45} in the mouse mammary gland in vitro and in vivo. However, unlike our results, they observed that PRL at 100 ng/mL improves the migration of T CD4+, T CD8+, B cells, macrophages, monocytes, and neutrophils during the mammary gland development. This effect of PRL upon the immune cells supports the hypothesis that the hyperprolactinemic environment prevalent along pregnancy has deep effects in the maternal immune system to maintain an anti-inflammatory environment compatible with tolerance to fetus.

Gomez-López et al\textsuperscript{29} previously reported selective chemotaxis of B cells in amnion extract tissues from term labor, while the recruitment of T cells, monocytes, and NK cells was driven by chorionic deciduial extract tissues. On the other hand, during vaginal term delivery, the participation of T cells, monocytes, and NK cells was driven by chorionic deciduial extract tissues. In our study, lymphocytes T CD4+ were the responsive subpopulation to amnion-conditioned media in response to LPS. Interestingly, T cells are the second most abundant population, after neutrophils, in amniotic fluid from women with spontaneous preterm labor and intra-amniotic infection.\textsuperscript{46} Besides, Frascoli et al\textsuperscript{49} found the important role of fetal T cells from preterm labor, which exerts a strong pro-inflammatory response to maternal antigens, furthermore, the authors demonstrate that T cells can be activated in vitro to produce INFγ and TNF-α, and eventually induce the myometrial contractility.

In summary, illustrated in Figure 6, we propose that in vitro LPS challenge of fetal membranes turn on the inflammatory response, lead in part by the differential chemokine secretion. Although amnion secretes MCP-1, MIP-1α and PECAM, the chorioamniotic membranes is the main responsive region after LPS stimulation, consequently, leukocyte migration, mainly T cells, increases (1). Consequently, leukocyte migration (mainly T cells) of amnion increases (2). We propose that PRL exerts a selective anti-inflammatory effect, modifying the chemotactic capacities of each compartment of fetal membranes, enhancing the intrinsic immunoregulatory properties of the amnion, regulating the traffic of T cells to the amniotic cavity, whose potential inflammatory response compromises local immunological tolerance and puts the normal continuation of pregnancy at risk.

In conclusion, PRL modifies the in vitro chemotactic capacities in fetal membranes, allowing us to speculate that this hormone exerts a protective effect that improves the immune response during adverse conditions such as infection, and supports the key role of PRL to maintain the local immune-privilege during pregnancy at the maternal-fetal interface.

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**CONFLICT OF INTEREST**

We declare no conflict of interest that could be perceived as impacting our impartiality to the informed investigation.

**DATA AVAILABILITY STATEMENT**

Taking into consideration that in this study, we used biological samples from pregnant women, demographics sensitive data of the patients that participate in this study are not publicly available due to privacy or ethical restrictions.

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