




## ARTICLE

# Trophoblast-secreted soluble-PD-L1 modulates macrophage polarization and function

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**Abstract**

Decidual macrophages are in close contact with trophoblast cells during placenta development, and an appropriate crosstalk between these cellular compartments is crucial for the establishment and maintenance of a healthy pregnancy. During different phases of gestation, macrophages undergo dynamic changes to adjust to the different stages of fetal development. Trophoblast-secreted factors are considered the main modulators responsible for macrophage differentiation and function. However, the phenotype of these macrophages induced by trophoblast-secreted factors and the factors responsible for their polarization has not been elucidated. In this study, we characterized the phenotype and function of human trophoblast-induced macrophages. Using *in vitro* models, we found that human trophoblast-educated macrophages were CD14<sup>+</sup>CD206<sup>+</sup>CD86<sup>-</sup> and presented an unusual transcriptional profile in response to TLR4/LPS activation characterized by the expression of type I IFN- $\beta$  expression. IFN- $\beta$  further enhances the constitutive production of soluble programmed cell death ligand 1 (PD-L1) from trophoblast cells. PD-1 blockage inhibited trophoblast-induced macrophage differentiation. Soluble PD-L1 (sPD-L1) was detected in the blood of pregnant women and increased throughout the gestation. Collectively, our data suggest the existence of a regulatory circuit at the maternal fetal interface wherein IFN- $\beta$  promotes sPD-L1 expression/secretion by trophoblast cells, which can then initiate a PD-L1/PD-1-mediated macrophage polarization toward an M2 phenotype, consequently decreasing inflammation. Macrophages then maintain the expression of sPD-L1 by the trophoblasts through IFN- $\beta$  production induced through TLR4 ligation.

**KEYWORDS**

IFN- $\beta$ , LPS, macrophage, PD1, soluble PD-L1, Trophoblast

## 1 | INTRODUCTION

Pregnancy exemplifies a unique immunologic condition in which cells at the maternal-fetal interface create a delicate balance between the support of fetal growth and development, tolerance to paternal antigens, and appropriate responses to infection if it occurs.<sup>1</sup> This microenvironment is dynamic and continuously adjusts to the different stages of fetal development. As such there is an active process of adaptation

and modulation from both the maternal and fetal sides.<sup>2</sup> The success of pregnancy depends on the appropriate communication between the fetal trophoblast and the immune cells present at the maternal-fetal interface including macrophages (reviewed in Zhang et al.<sup>3</sup> and Mor and Cardenas<sup>4</sup>).

Macrophages comprise 20–25% of the total leukocyte population in early pregnancy, and their presence is maintained throughout pregnancy. Macrophages are classically categorized into M1

and M2 subtypes<sup>5</sup> with M1 macrophages geared toward clearing infections (proinflammatory) and M2 macrophages better poised for tissue remodeling and repair (anti-inflammatory). This classification follows the TH1 and TH2 nomenclature used to identify pro- and anti-inflammatory T cells.<sup>6,7</sup> Despite the nomenclature similarities, no master regulator that directs macrophage differentiation has been described, emphasizing individual cell plasticity in the myeloid lineage rather than discrete cell types.<sup>8</sup> Decidual macrophages are differentiated from monocytes derived from the bone marrow,<sup>3,8-11</sup> which migrate from the bloodstream to the uterus during the pre-implantation period and differentiate into decidual-specific macrophages upon exposure to this local microenvironment.<sup>12-15</sup> Decidual macrophages have a high degree of plasticity that allows them to change their phenotypes based on the signals present at the implantation site. During the pre-implantation period, macrophages are mainly the M1 phenotype,<sup>16</sup> they change to M2 phenotype following trophoblast attachment and invasion and then revert to M1 phenotype at the time of delivery.<sup>3,17</sup> Inappropriate macrophage polarization during a specific developmental stage can have a detrimental effect on fetal development and pregnancy outcome.<sup>3</sup> Our current knowledge on the factors regulating macrophage polarization at the implantation site is however still limited.

The trophoblasts represent the first point of contact between the blastocyst and maternal decidua and play an active role in shaping the immunologic milieu at the implantation site.<sup>1</sup> They have the ability to sense and respond to their microenvironment through the expression of pattern recognition receptors such as TLRs, which can recognize specific molecular patterns released from the local cells including decidual stromal cells and immune cells, known as damage associated molecular patterns (DAMS) or molecules released by commensal or pathogenic bacteria (pathogen associated molecular patterns, PAMS).<sup>18-21</sup> Trophoblast-secreted factors are able to recruit and modulate the differentiation and function of immune cells at the maternal-fetal interface throughout pregnancy.<sup>4</sup> We and others have shown that trophoblast-secreted factors present in the trophoblast conditioned media (CM) promote T cell differentiation into regulatory T cells (Tregs) and CD14<sup>+</sup> monocytes into decidual macrophages.<sup>12,14,22</sup> Indeed, we demonstrated that trophoblast CM could induce monocyte differentiation into macrophages characterized by gain of CD14 and CD16 surface expression and enhanced production of chemokines such as IL-10 and CXCL10 as well as increased capacity for phagocytosis.<sup>14</sup> We designated these macrophages as trophoblast-educated macrophages (TEMs). The functional properties of these TEMs and the specific factors in the trophoblast CM that are required for their differentiation have, however, not been elucidated.

Thus, the objectives of this study were to further characterize the role of trophoblast-secreted factors in decidual macrophage differentiation by performing a phenotypic characterization of TEMs and transcriptional assessment of their response to TLR4 stimulation. In addition, we evaluated the potential factor(s) produced by the trophoblast cells responsible for macrophage polarization. Our findings show that

TEMs are CD14<sup>+</sup>/CD206<sup>+</sup>/CD86<sup>-</sup> and present a unique transcriptional profile in response to TLR4/LPS stimulation dominated by type I IFN-responsive pathways. Moreover, we demonstrate that soluble PD-L1 (sPD-L1) secreted by trophoblast cells and present in the blood of pregnant women, is responsible for TEM polarization. These findings provide a better understanding of the unique immune regulatory network that is present at the implantation site and necessary for the success of pregnancy.

## 2 | MATERIAL AND METHODS

### 2.1 | Ethics statement

Studies with human blood monocytes were approved by the institutional review board (IRB) committee of Yale University with no written consent requirement (#2000021607). Monocytes were obtained from healthy individuals eligible for single donor platelet apheresis in a blood bank setting. Investigators had no access to any personal information. All the samples evaluated had no identifiers.

### 2.2 | Reagents, antibodies, and cell lines

The cell lines used in the experiments were the first trimester trophoblast cell line Swan 71 established in our laboratory (21) and 3A cells obtained from ATCC.<sup>23,24</sup> Cells were cultured in RPMI media supplemented with 10% FBS, 1000 U/ml penicillin, 100 ug/ml streptomycin, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 nM nonessential amino acids, and 1 mM sodium pyruvate, and maintained at 37°C with 5% CO<sub>2</sub>.

LPS isolated from *Escherichia coli* (O111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The magnetic multiplex beads were obtained from Bio-Rad Laboratories (Hercules, CA, USA), and they were precoated with the antibodies recognizing IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 (p70), IL-17, G-CSF, GM-CSF, IFN- $\gamma$ , MCP-1, MIP-1 $\alpha/\beta$ , TNF- $\alpha$ , CCL5 or RANTES, vascular endothelial growth factor (VEGF), CXCL10, and growth-regulated oncogene-alpha (GRO- $\alpha$ ).

### 2.3 | Patient recruitment and serum sample collection

#### 2.3.1 | Study population

The description of the patients' population, recruitment, and characteristics has been previously described<sup>25</sup> as following:

This study included 506 serum samples collected between 3 and 13 wk (25 to 96 d) of gestation. In order to include enough samples from the possible earliest time points in pregnancy, samples were collected from two prospectively followed pregnancy cohorts—women undergoing in vitro fertilization (IVF) treatment ( $n = 40$ ) and women with natural conceptions ( $n = 102$ ). The samples from the IVF cohort included 83 data points for normal pregnancies (2–6 data points per

individual sample). The natural conception cohort included 270 data points for normal pregnancies (3–4 data points per individual sample).

A limitation of this cohort was the lack of racial diversity. The majority of the patients were Caucasian because of the country of enrollment (Denmark).<sup>26</sup> Details for each cohort are described below.

**IVF cohort.** Recruitment of IVF patients and storage of samples were approved by the Yale institutional IRB with no written consent requirement (#2000021607). The study was deemed to have minimal harm to patients thus only verbal consent was requested. The investigators had no access to any personal information. The coded serum samples were provided to the investigators without any patient information. The patients eligible for participation in this study included those who were between 18 and 44 yr old and underwent fresh or frozen day 3 or day 5 (blastocyst) embryo transfer during the period of October 2017 to July 2018. Exclusion criteria were patients with chronic autoimmune disease (such as lupus, thyroid antibodies, ulcerative colitis, or Crohn's disease), diabetes and hypertension requiring medication treatment, endometriosis confirmed by laparoscopy, or current illness with inflammatory processes. Patients were also excluded if they had prior pregnancy losses, unless the tissue from the loss had undergone genetic testing and was determined to be chromosomally abnormal. Patients were asked to participate at the time of embryo transfer. Blood was collected by venipuncture into 10 mL vacutainer tubes at the time showing the first positive  $\beta$ -hcg, then 8–12 d after embryo transfer, and then every 48 h until an intrauterine pregnancy was confirmed using transvaginal ultrasound. Samples were left at room temperature for 60 min to allow for clotting and then centrifuged (Thermo Scientific Sorvall ST 16, Waltham, MA, USA) at 3000 rpm for 10 min at room temperature. Serum was aliquoted into 1.5 mL polypropylene RNase- and DNase-free microcentrifuge tubes and stored in  $-80^{\circ}\text{C}$  freezers until ready for testing.

**Naturally conceived cohort.** The natural conception cohort was part of a larger Danish prospective early pregnancy cohort (the PEP cohort) including women recruited through online advertisement in the period of 2016–2017. The characteristics of the patient population recruited in the study are described in a previous publication.<sup>26</sup> In short, healthy women at the age of 18 yr or older with a singleton pregnancy and able to understand and sign written consent were eligible for participation. Exclusion criteria included history of recurrent pregnancy losses ( $\geq 3$  losses, including biochemical pregnancies), any type of assisted reproductive techniques, uterine or tubal abnormalities assessed at the first visit, and ongoing substance abuse. The patient follow-up with serial blood draws and transvaginal ultrasound started as soon as the women expressed interest after a positive urine pregnancy test and continued every 2 wk until completion of the first trimester (11–14 wk of gestation). Blood was collected by venipuncture into vacutainer separator tubes (BD Diagnostics, Franklin Lakes, NJ, USA), and allowed to clot for 15 min at room temperature and then centrifuged (Hettich Rotina 380 R, Andreas Hettich GmbH, Tuttlingen, Germany) at 3500 rpm at  $5^{\circ}\text{C}$  for 10 min. Serum was aliquoted into the same plastic vials as those from the IVF cohort and stored in  $-80^{\circ}\text{C}$  freezers until ready for testing. All specimens from both cohorts were transported in liquid nitrogen to Yale University.

## 2.4 | Human primary trophoblast isolation and culture

Human primary trophoblast cells were isolated from first trimester elective terminations as previously described.<sup>23</sup> A signed written consent form was obtained from the patients. The use of placental tissues, specimens and consent forms was approved by the Yale University Human Investigation Committee (#2000021607). The tissue specimen was collected in cold, sterile PBS and immediately transported to the laboratory for cell culture preparation. Briefly, first trimester placental villous tissues were cut and digested in PBS supplemented with 0.25% Trypsin (Gibco, Grand Island, NY, USA) for 10 min at  $37^{\circ}\text{C}$  with gentle agitation. An equal volume of 10% FBS (Gibco) and DMEM (Gibco) was added to inactivate the trypsin. The supernatant was collected and centrifuged at 1500 rpm at room temperature for 10 min. The pellet was resuspended in 5 ml DMEM media supplemented with 10% FBS. This suspension was laid over lymphocyte separation media (ICN Biomedicals, Inc., Aurora, OH, USA) and centrifuged at 2000 rpm for 20 min. The interface containing the trophoblast cells was collected and centrifuged at 1500 rpm for 10 min. Cells were resuspended in DMEM with 10% FBS and then plated on a 6-well plate to grow.

## 2.5 | Preparation of trophoblast conditioned media

First trimester trophoblast Swan 71 cells<sup>27</sup> or first trimester trophoblast primary cell cultures were plated at  $5 \times 10^5$  cells/100 mm dish with DMEM-F12 media containing 10% FBS and allowed to attach overnight. Media were then changed to DMEM-F12 media containing 1% FBS and incubated for additional 48 h. Cell supernatant was collected, clarified, aliquoted, and stored at  $-80^{\circ}\text{C}$  until use.

## 2.6 | Monocyte isolation

CD14<sup>+</sup> monocytes were isolated from concentrated leukocytes from nonpregnant females between the ages of 25 and 35 (see Section 2.1, Ethics Statement). CD14<sup>+</sup> monocyte isolation was performed as previously described.<sup>14,28</sup> Briefly, leukocytes were diluted 1:1 in PBS, laid onto lymphocyte separation medium and centrifuged without brake at 2000 rpm at room temperature for 20 min. The mononuclear layer was then resuspended in PBS and centrifuged at 800 g at room temperature for 10 min. The pellet was then resuspended in Easy Sep buffer (0.5% BSA, 2 mM EDTA acid, pH 7.2), and the human CD14<sup>+</sup> monocytes were purified by positive selection using the monocyte isolation kit II (EasySep, Vancouver, British Columbia, Canada) according to the manufacturer's protocol. The purity of the CD14<sup>+</sup> cells was  $>95\%$  as determined by flow cytometry.

## 2.7 | Macrophage differentiation

Freshly isolated CD14<sup>+</sup> cells were plated at  $2 \times 10^6$  cell/well in DMEM/F-12 media containing 1% FBS. Cells were then treated with or without 50% CM from the first trimester trophoblast cell line Sw.71, or 25 ng/mL GM-CSF, or 50 ng/mL M-CSF at  $37^{\circ}\text{C}$  in a humidified atmosphere (5% CO<sub>2</sub>) for a total of 7 d; media were refreshed once

every 2 d as previously described.<sup>14</sup> The macrophages treated with GM-CSF and M-CSF were designated as M1-like and M2-like, respectively, and macrophages differentiated with trophoblast CM were designated as TEMs. For the LPS studies, the macrophages differentiated for 6 d were treated with 10 ng/ml LPS for 24 h, and the cells and supernatants were collected. For the PD-1 blocking studies, the purified CD14<sup>+</sup> cells were treated with an anti-PD-1 mAb for 7 d in the presence of trophoblast CM or GM-CSF or M-CSF. The anti-PD-1 mAb treatment was refreshed once every 2 d. At day 7 of the study, cells and supernatants were collected and assessed for markers of differentiation by flow cytometry and for cytokines/chemokines by SimplePlex (ELLA) and Luminex.

## 2.8 | Flow cytometry

The cells collected from differentiation cultures were analyzed by flow cytometry (FACSCalibur, Beckton Dickinson, Franklin Lakes, NJ, USA). PE-conjugated anti human-CD14 and FITC-conjugated anti human-CD16 Abs were purchased from eBioscience (San Diego, CA, USA) and used at a 1:50 dilution. The FITC-conjugated anti-human CD206 antibody (Biolegend, San Diego CA, USA), phycoerythrin (PE)-conjugated anti-human CD14 antibody (Biolegend), and PE-Cy5-conjugated anti-human CD86 antibody (Biolegend) were used following the instructions from the manufacturer. FACS data were analyzed by FlowJo (Treestar, Ashland, OR, USA).

## 2.9 | Cytokine and sPD-L1 analysis evaluation by ELLA

The cytokine profile of supernatants from the differentiated macrophages with or without LPS stimulation was determined using the Simple Plex immunoassay system (ELLA, Protein Simple, San Jose, CA, USA) as previously described.<sup>29</sup> Briefly, 50  $\mu$ l of sample was added to sample inlet ports on a cartridge, and each sample from a single sample inlet port was split into multiple parallel channels. Each channel was specific for one particular analyte and subjected to a typical sandwich immunoassay protocol. The entire immunoassay procedure was automated, and the analyzed results were obtained using the manufacturer-encoded calibration curves.

## 2.10 | Quantitative RT-PCR

RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA, USA) and an equal amount of RNA (1  $\mu$ g) was used for cDNA synthesis using iScript Reverse Transcription Supermix (Bio-Rad Laboratories). The cDNA was diluted to 1:5 in nuclease-free water, and 5  $\mu$ l were used in the PCR. The gene-specific primers and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) were added to the cDNA and run on the CFX96 C1000 system Quantitative PCR machine (Bio-Rad Laboratories). The primer sequences were previously described.<sup>30</sup> The amount of target relative to a calibrator was computed by  $2^{-\Delta\Delta CT}$ , and the housekeeping gene  $\beta$ -actin (ACTB) or GAPDH was used for normalization.

## 2.11 | PCR array

RNA was extracted using the RNeasy kit. RNA concentration and purity were assessed using spectrophotometric analyses of 260/280 ratios, and only samples with values of 1.8 or above were used for analysis. For quantitative analysis of messenger RNA (mRNA), 1  $\mu$ g of RNA from each sample was reverse-transcribed using the RT<sup>2</sup> First Strand Kit (Qiagen) as per manufacturer's protocol and used for the RT<sup>2</sup> Profiler PCR Human Toll-Like Receptor Signaling Pathway Array (cat# PAHS-108Z) as directed to run on a Bio-Rad CFX96 cyclor. Fold change analysis was performed using Qiagen's GeneGlobe Data Analysis Center (<https://dataanalysis.qiagen.com/pcr/arrayanalysis>).

## 2.12 | Western blot

Protein extraction was performed using Cell Lysis Buffer (Cell Signaling, Danvers, MA, USA), and the total protein concentration was determined using the Pierce BCA Assay Kit (Thermo Fisher, Rockford, IL, USA). Equivalent amount of total protein was boiled for 5 min, separated on 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Perkin Elmer, Shelton, CT, USA). Membranes were blocked with 5% nonfat milk for 1 h at room temperature and incubated with primary antibodies at 4°C overnight. The membranes were washed and incubated with corresponding HRP-labeled secondary antibodies at room temperature for 2 h. Blots were imaged with the Kodak Image Station 400 (Eastman Kodak, Rochester, NY, USA).

## 2.13 | Statistical analysis

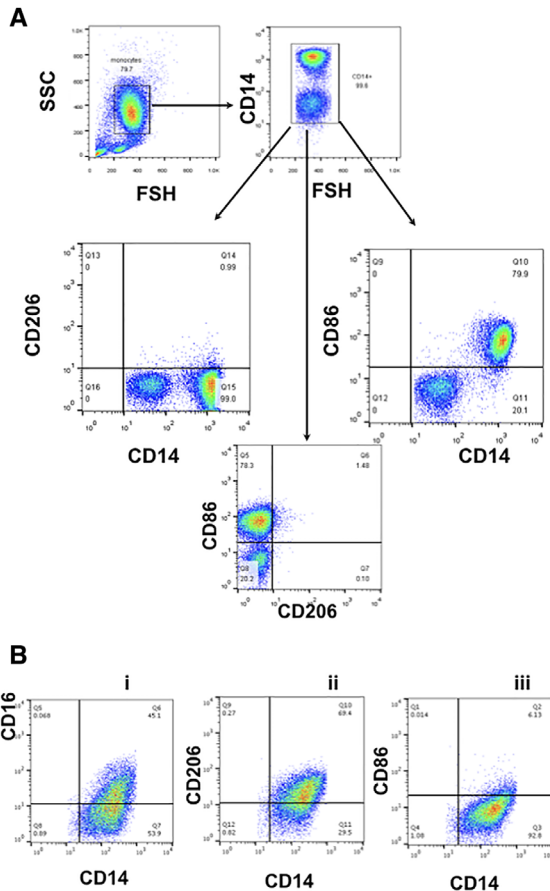
Statistical analyses were performed using the Statistical Package for Social Science (SPSS, IBM, New York, NY, USA) for windows and Prism software, version 5 (GraphPad, San Diego, CA, USA). Differences between two groups were analyzed using Student's *t*-test. The differences among multiple groups were analyzed by 1-way ANOVA and  $\chi^2$  test. *P*-values less than 0.05 were considered significant. All the experiments were done in triplicate and a minimum of three independent experiments.

# 3 | RESULTS

## 3.1 | Induction of a unique population of macrophages by trophoblast CM

Previously we reported that exposure of CD14<sup>+</sup> peripheral blood monocytes to trophoblast CM induced their differentiation into decidua-like-macrophages. As stated above, we designated them as TEMs.<sup>14</sup> Our first objective was to further characterize the phenotype of these macrophages differentiated by trophoblast-secreted factors in terms of their heterogeneity. Figure 1 shows flow cytometry strategy for the characterization of monocytes isolated from peripheral blood. As previously shown, monocytes are a heterogeneous population,<sup>31</sup> where classical monocytes have high CD14 (CD14<sup>high</sup>) and nonclassical monocytes have low CD14 (CD14<sup>dim</sup>),<sup>32,33</sup> and they





**FIGURE 1** Phenotype of peripheral blood monocytes before and after differentiation stimulated by trophoblast conditioned media (CM). A. Gating strategy was set up for characterization of circulating monocytes. Isolated peripheral blood CD14<sup>+</sup> monocytes express CD14 and CD86 but not CD206 (CD14<sup>+</sup>/CD206<sup>-</sup>/CD86<sup>+</sup>). B. Freshly isolated CD14<sup>+</sup> monocytes were exposed to first trimester trophoblast CM for 6 d and then analyzed by flow cytometry for CD14, CD86, CD206, and CD16. Following treatment with trophoblast CM, monocytes/macrophages maintained CD14 expression, and gained CD16 and CD206, but lost CD86 (CD14<sup>+</sup>/CD16<sup>+</sup>/CD206<sup>+</sup>/CD86<sup>-</sup>). Representative figure of 6 independent experiments with each done in triplicate

are mainly the M1 (inflammatory) type<sup>34</sup> characterized by being CD86<sup>+</sup>/CD206<sup>-</sup> (Fig. 1A). The CD14<sup>dim</sup> population is double negative (CD86<sup>-</sup>/CD206<sup>-</sup>) (Fig. 1A). We isolated the CD14<sup>+</sup> monocytes from PBMCs by magnetic beads and exposed them to trophoblast CM for 7 d and evaluated their phenotype by flow cytometry. Exposure of these CD14<sup>+</sup> monocytes to trophoblast CM promoted their differentiation into macrophages that maintained their CD14 expression and further gained CD16 expression (CD14<sup>+</sup>/CD16<sup>+</sup>) (Fig. 1B).<sup>14</sup> Furthermore, they gained CD206 and lost CD86 expression, suggesting a differentiation toward a M2 phenotype that resembled decidual macrophages (CD14<sup>+</sup>/CD206<sup>+</sup>/CD86<sup>-</sup>)<sup>9,11,35-37</sup> (Fig. 1B). Isotypes for the different markers are shown in Supporting Information Fig. S1. Accordingly, these in vitro results suggest that the placenta, more specifically trophoblast-secreted factors play an important role in the process of differentiation of local macrophages.

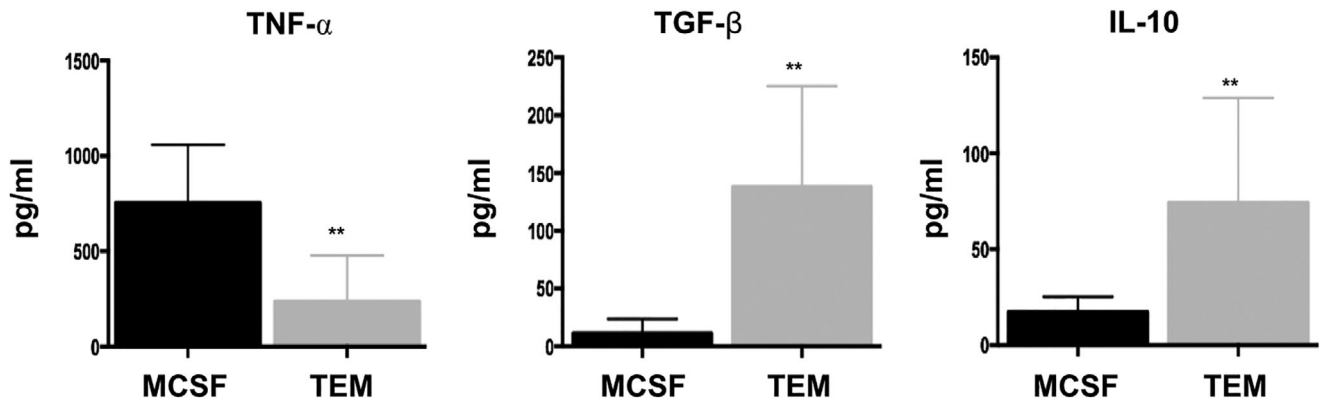
### 3.2 | Differential response of TEMs to LPS

The basal cytokine profile and the cytokine profile in response to stimulus reflect the functional differentiation status of monocytes/macrophages.<sup>38-40</sup> Thus, to determine the functional consequence of differentiation stimulated by trophoblast CM, we first analyzed the basal cytokine secretion profile of TEMs and compared them to the M-CSF differentiated macrophages (MDMs), which had a classical M2-like phenotype. Thus, CD14<sup>+</sup> monocytes were differentiated in the presence of trophoblast CM or M-CSF for 5 d. To determine the factors secreted by the differentiated cells and not the CM, the cultures were replaced with fresh media for additional 24 h and collected for cytokine characterization. Interestingly, TEMs presented a distinct secretory cytokine profile characterized by a lower level of TNF- $\alpha$  and higher levels of TGF- $\beta$  and IL-10 than that in MDMs (Fig. 2).

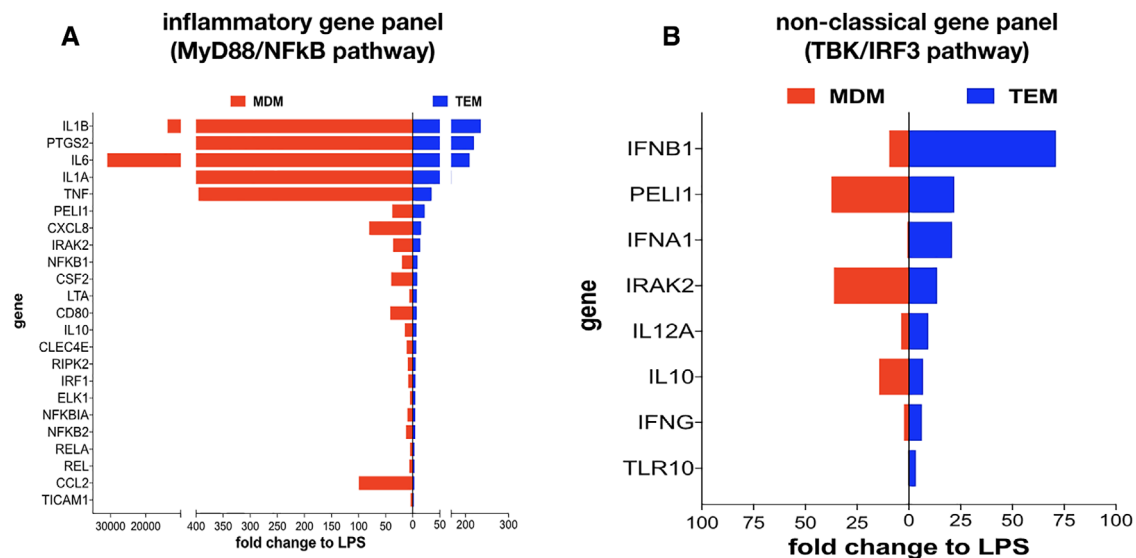
Next, we characterized the functional response of TEMs and MDMs to TLR stimulation with a focus on TLR4 signaling using LPS. Thus, upon 7 d of differentiation by trophoblast CM (for TEMs) or M-CSF (for MDMs) treatment, the cells were exposed to LPS (10 ng/ml) for 8 h and their response was first characterized by determining the gene profile of cytokines and chemokines using a cytokine/chemokine PCR array. We observed distinct responses from the two types of macrophages. Whereas the MDM's response to LPS was associated with increased mRNA levels of IL-1 $\beta$ , PTGS2, IL-6, IL-1 $\alpha$ , and TNF- (Fig. 3A), the TEM's response revealed a predominantly nonclassical profile characterized by an increase in type I IFNs (Fig. 3B). In order to confirm the results from the PCR array, we performed RT-qPCR using mRNA samples from TEMs and MDMs treated with LPS (10 ng/ml) for 8 h. When we compared the magnitude of the response between the two cell types, TEMs presented a significant lower expression of TNF- $\alpha$ , IL-6 but a much higher expression of the chemokine CCL5 than MDMs (Fig. 4). This profile is consistent with the results from the PCR array (Fig. 3).

### 3.3 | Activation of TLR4/TBK/IRF3 pathway in TEMs

We then sought to characterize the differential pathways activated in TEMs and MDMs (classical M2 macrophages) in response to TLR4 ligation by LPS. TLR4 ligation by LPS has been shown to induce two distinct pathways: the classical MyD88-NF $\kappa$ B signaling cascade leading to the production of proinflammatory cytokines<sup>41</sup> and the TBK/IRF3 pathway leading to the production of type I IFNs.<sup>42,43</sup> We hypothesized that in the TEMs, TLR4 ligation by LPS preferentially would signal through the nonclassical TBK/IRF3 pathway because we did not observe a favored increase in NF- $\kappa$ B-regulated proinflammatory cytokines. To test this hypothesis, TEMs and MDMs were treated with LPS (10 ng/ml) and samples were collected at 15, 30, 60, 240, and 480 min posttreatment. We first determined the effect of LPS on the MyD88/NF $\kappa$ B pathway by evaluating I $\kappa$ B $\alpha$ , an inhibitor of p65 nuclear translocation.<sup>44</sup> A decrease in I $\kappa$ B $\alpha$  would suggest its degradation and consequently p65 nuclear translocation and the induction of p65-induced gene transcription. As we hypothesized, we did not observe I $\kappa$ B $\alpha$  degradation in the TEMs upon TLR4 ligation with



**FIGURE 2** Differential cytokine profile between M-CSF polarized macrophages (MDMs) and trophoblast-polarized macrophages (TEMs). Freshly isolated CD14<sup>+</sup> monocytes were differentiated with trophoblast conditioned media (CM; TEM) or M-CSF (MDM) for 6 d. Following differentiation their basal cytokine profile was quantified. TEMs presented a distinct cytokine profile compared to MDMs characterized by lower secretion of TNF- $\alpha$ , but higher secretion of TGF- $\beta$  and IL-10. Data presented as mean  $\pm$  SD and are shown for 6 independent experiments with each done in triplicate. \*\**P* = 0.01

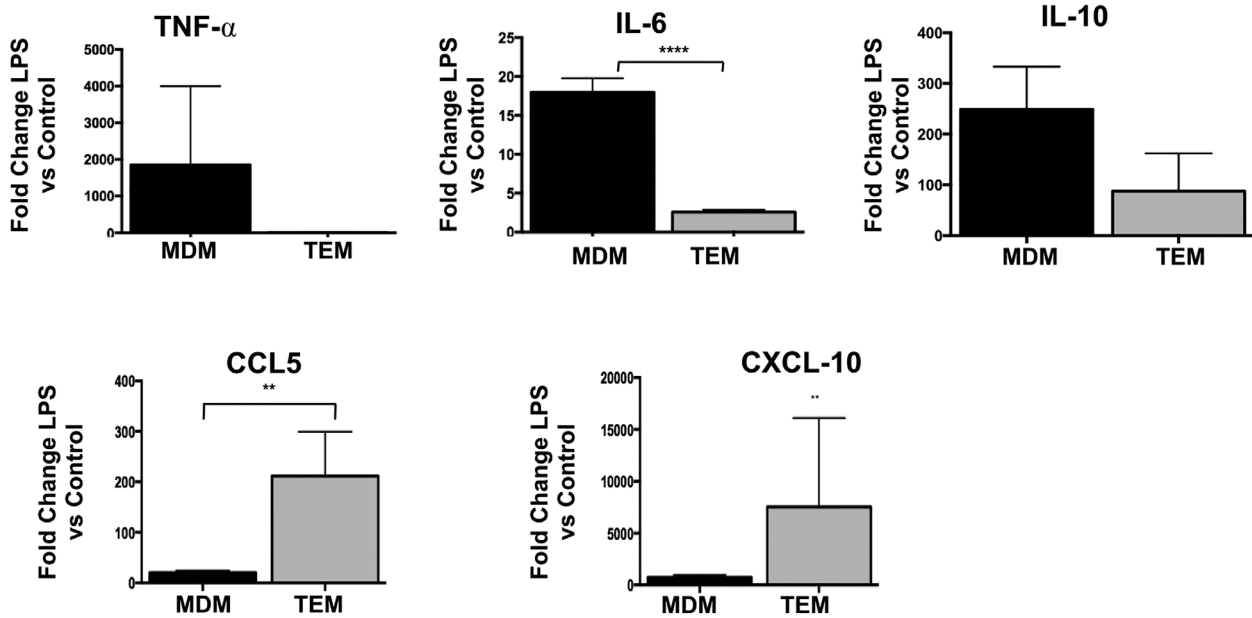


**FIGURE 3** Differential response to TLR4 ligation by LPS. Freshly isolated CD14<sup>+</sup> monocytes were differentiated with trophoblast conditioned media (CM; trophoblast-educated macrophages; TEMs) or M-CSF (MDMs) for 6 d. Following differentiation cells were exposed to LPS for 8 h and their response to LPS was characterized by cytokine PCR array. **A.** LPS treatment induced a higher mRNA expression of proinflammatory cytokines in MDMs than in TEMs. Fold changes in mRNA expression were calculated for each group treated with LPS in relation to their respective control (treated with vehicle) **B.** Type I IFN- $\beta$  and IFN- $\alpha$  and associated genes were highly induced by LPS treatment in TEMs compared to MDMs. Fold changes in mRNA expression were calculated for each group treated with LPS in relation to their respective control (treated with vehicle)

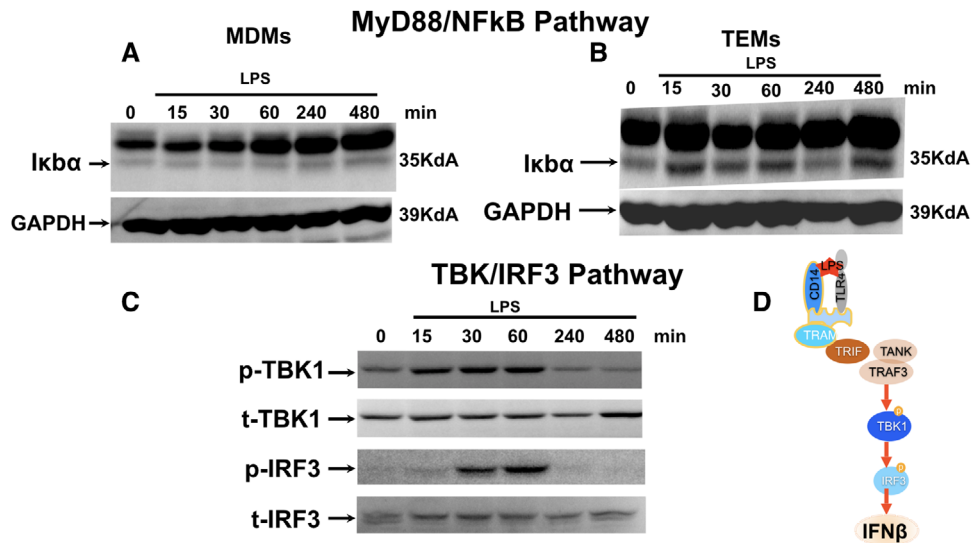
LPS (Fig. 5B). In contrast, we observed low levels of I $\kappa$ B $\alpha$  in MDMs, suggestive of its degradation (Fig. 5A). These findings suggest that in TEMs, LPS may promote the activation of the nonclassical pathway TBK/IRF3 pathway. To confirm it, we evaluated the activation status of the TBK/IRF3 pathway in TEMs by determining the phosphorylation status of TBK after LPS stimulation. We observed an increase in the levels of phosphorylated TBK (pTBK) within 15 min of exposure to LPS. In addition, we also observed an increase in the levels of phosphorylated IRF3 (pIRF3), a transcription factor downstream of TBK and a major regulator of type I IFN expression.<sup>43,45,46</sup> Total TBK and IRF3 expression was not affected by LPS treatment (Fig. 5C). The observed changes on pTBK and pIRF3 were transient with their

levels resuming to baseline by 240 min (Fig. 5C). On the other hand, no changes of pIRF3 or pTBK were observed in MDMs (data not shown). Then we evaluated the mRNA expression of IFN- $\alpha$  and IFN- $\beta$  in TEMs following LPS treatment. In accordance, TLR4 ligation by LPS in TEMs resulted in an increase in mRNA levels of IFN- $\alpha$  and IFN- $\beta$  (Fig. 6A) as well as an increase in the antimicrobial factors beta-defensin-1, -2, and SLP1 (Fig. 6B). These findings demonstrate that in TEMs, LPS promotes the expression of type I IFN through the TLR4/ TBK/ IRF3 pathway.

To further confirm the involvement of TBK and IRF3 in TLR4-induced type I IFN response in TEMs, we exposed TEMs to LPS (10 ng/ml) in the presence or absence of BX795 (10 nM).<sup>47</sup> BX795 is a



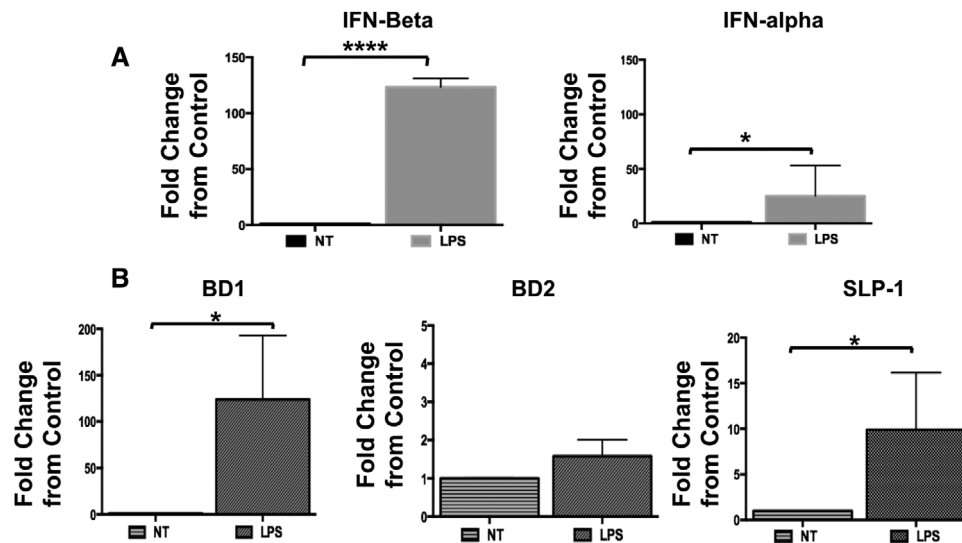
**FIGURE 4** Validation of the differential cytokine profile in response to TLR4 ligation by LPS. Freshly isolated CD14<sup>+</sup> monocytes were differentiated with trophoblast conditioned media (CM; trophoblast-educated macrophages; TEM) or M-CSF (MDM) for 6 d. Following differentiation cells were exposed to LPS for 24 h and mRNA of each cytokine was quantified by qPCR using specific primers. Data was presented for each cytokine as fold changes in mRNA expression calculated for each group treated with LPS in relation to their respective control (treated with vehicle). Data presented as mean + SD and are shown for 6 independent experiments with each done in triplicate. \*\* $P = 0.01$ ; \*\*\*\* $P = 0.001$



**FIGURE 5** LPS-TLR4 ligation in trophoblast-educated macrophages (TEMs) is associated with the activation of the MyD88 independent pathway TBK/IRF3. Freshly isolated CD14<sup>+</sup> monocytes were differentiated with trophoblast conditioned media (CM; TEM) or M-CSF (MDM) for 6 d followed by treatment with LPS (10 ng/ml) for 15, 30, 60, 240, and 480 min. **A.** Effect of LPS treatment on MyD88/NF $\kappa$ B pathway was determined in M-CSF polarized macrophages (MDMs) by analyzing levels of I $\kappa$ B $\alpha$  by Western blot analysis. Note the low levels of I $\kappa$ B $\alpha$  expression in MDM. **B.** Effect of LPS treatment on MyD88/NF $\kappa$ B pathway was determined in TEMs by analyzing levels of I $\kappa$ B $\alpha$  by Western blot analysis. Note the continuation of high expression of I $\kappa$ B $\alpha$  in the presence of LPS treatment. **C.** Effect of LPS treatment on TBK/IRF3 pathway was determined by analyzing phosphorylation status of TBK and IRF3 by Western blot analysis. pTBK1 = phosphorylated TBK; tTBK1 = total TBK1. pIRF3 = phosphorylated IRF3; and t-IRF3 = total IRF3. Representative figure of 3 independent experiments

potent and specific inhibitor of TBK1 and blocks the phosphorylation, nuclear translocation, and transcriptional activity of IRF3, and hence, the production of IFN- $\beta$ .<sup>48</sup> We found that in the presence of BX795, LPS-induced phosphorylation of IRF3 was inhibited (Fig. 7A). More

importantly, we observed a significant suppression of LPS-induced IFN- $\alpha$  and IFN- $\beta$  expression in TEMs (Fig 7B). In addition, LPS-induced increase in CCL5, CXCL10, and the anti-microbial factors BD1, BD2, and SLP-1 were also curtailed in the presence of BX795 (Fig. 8).



**FIGURE 6** LPS-TLR4 ligation promotes type I IFN and beta defensins in trophoblast-educated macrophages (TEMs) Freshly isolated CD14<sup>+</sup> monocytes were differentiated with trophoblast conditioned media (CM; TEM) for 6 d. Following differentiation cells were treated with LPS (10 ng/ml) for 8 h. mRNA expression of type I IFNs and the ISGs BD1, BD2, and SLP-1 was determined by qPCR. Data presented as mean + SD and are shown for 3 independent experiments with each done in triplicate. **A.** Type I IFN- $\alpha$  and INF- $\beta$  mRNA expression in TEMs treated with LPS. Fold changes in mRNA expression were calculated for each group treated with LPS in relation to their respective control (treated with vehicle). **B.** Expression of anti-microbial factors, beta defensins 1 and 2 (BD1 and BD2) and SLP-1 in TEMs treated with LPS. Fold changes in mRNA expression were calculated for each group treated with LPS in relation to their respective control (treated with vehicle).  $n = 6$  independent experiments and each one done in triplicates. Data presented as mean + SD and are shown for 6 independent experiments with each done in triplicate. \* $P = 0.05$ ; \*\*\*\* $P = 0.001$

Collectively, these data demonstrate that the dominant signaling in TEMs in response to LPS is the nonclassical TLR4/TBK1/IRF3/IFN pathway.

### 3.4 | The role of PD-1/PD-L1 pathway in trophoblast-induced macrophage polarization

Our next objective was to elucidate the potential factors produced by trophoblast cells responsible for the phenotype of TEMs. Programmed cell death-1 (PD-1) and its ligand (PD-L1) are key modulators of immune cell function and differentiation and have been suggested to play an important role in macrophage polarization and function.<sup>49</sup> During early pregnancy, PD-L1 expression is observed in syncytiotrophoblasts, cytotrophoblasts, and extravillous trophoblast cells,<sup>50</sup> indicating the involvement of trophoblast cells in modulating immune cells. Because we observed the expression of PD-1 in decidual macrophages,<sup>50</sup> we hypothesized that trophoblast cells would express and secrete PD-L1, which could be the key mediator of macrophage differentiation leading to the TEM phenotype. To test this hypothesis, CD14<sup>+</sup> monocytes were incubated with trophoblast CM in the presence or absence of a specific PD-1-blocking antibody, which would prevent the engagement of PD-L1 (secreted by trophoblast) on PD-1 expressed by CD14<sup>+</sup> monocytes and consequently block the activation of the PD-L1/PD-1 pathway. Our data showed that CD14<sup>+</sup> monocytes exposed to trophoblast CM with an isotype control IgG differentiated into CD14<sup>+</sup>/CD206<sup>+</sup>/CD86<sup>-</sup>; however, when the same trophoblast CM was added in the presence of PD-1 blocking antibody we observed a decrease in the CD206<sup>+</sup> cells and an increase

in the CD86<sup>+</sup> macrophages (Fig. 9B) that resembled the percentages of the CD86<sup>+</sup> and CD206<sup>+</sup> macrophages observed in the GM-CSF differentiated group, which were the classical M1 phenotype (Fig. 9C). These data suggest that by blocking PD-1, trophoblast CM fails to induce a M2 macrophage differentiation.

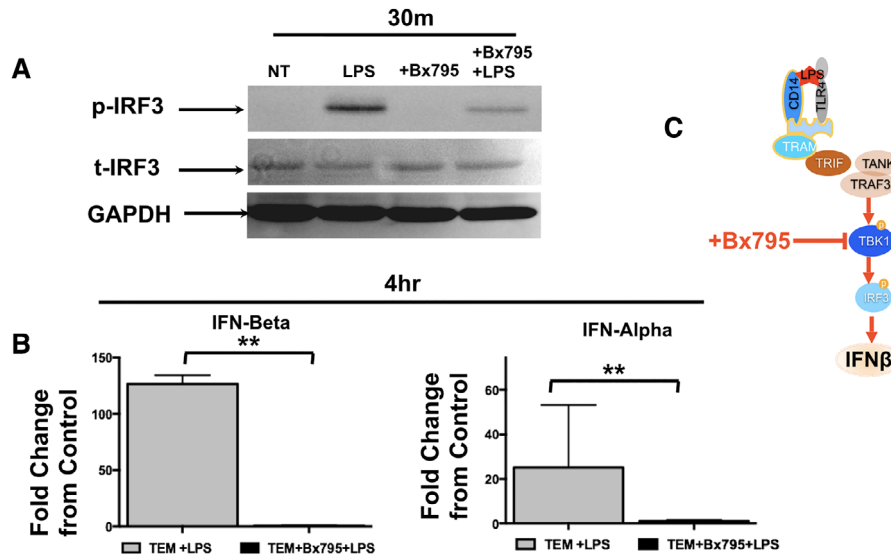
Furthermore, the response to LPS in the macrophages differentiated with trophoblast CM in the presence of PD-1 blocking antibody was reversed from the type I IFN expression status toward a proinflammatory cytokine profile with significantly higher levels of TNF- $\alpha$  and IL-6 expression (Fig. 10). This phenotype resembles M1-like macrophages, as demonstrated by the type of response observed with GM-CSF-differentiated macrophages (Fig. 10). To further prove the role of PD-1/PD-L1 pathway in macrophage polarization, we treated GM-CSF-differentiated macrophages (M1-like) with recombinant PD-L1 (PDL-1FC) and showed that PD-L1 was able to promote their polarization toward an M2 phenotype (CD14<sup>+</sup>CD206<sup>+</sup>) (Supporting Information Fig. S1).<sup>50</sup>

Taken together, these results demonstrate that by blocking PD-1 in the monocytes, we can prevent trophoblast-induced differentiation of CD14<sup>+</sup> monocytes into M2 macrophages, suggesting that the PD-1/PD-L1 signaling pathway is a major effector of trophoblast-induced macrophage polarization.

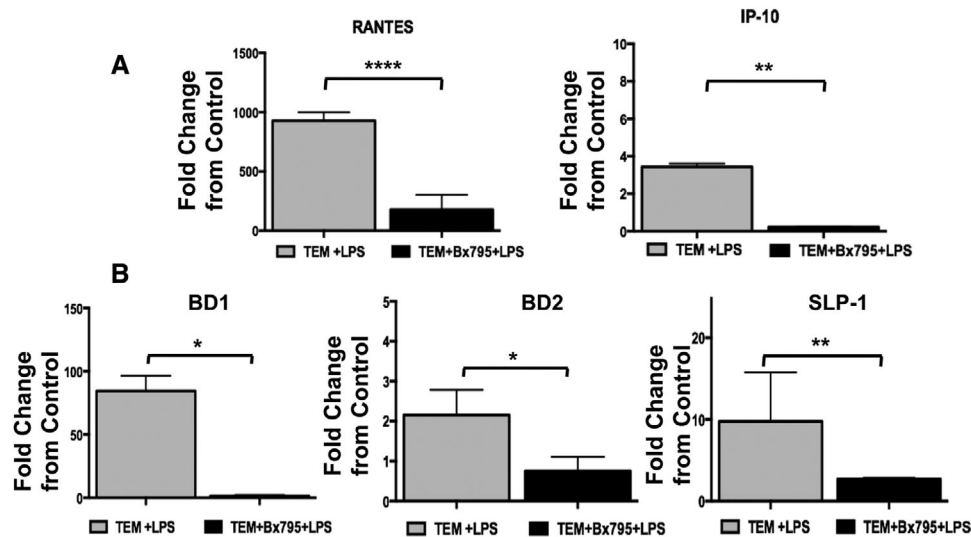
### 3.5 | Expression and secretion of sPD-L1 by trophoblast cells

The findings described above using trophoblast CM suggest that the effect on macrophage polarization is due to the secreted soluble form





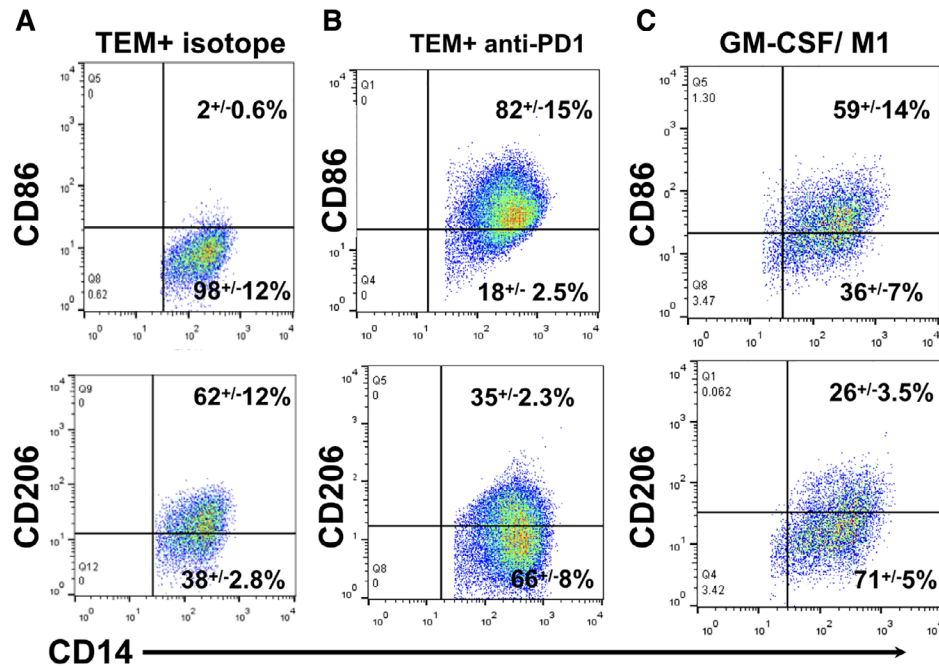
**FIGURE 7** TLR4 ligation by LPS promotes IFN- $\beta$  expression through the TBK-IRF3 pathway. Freshly isolated CD14<sup>+</sup> monocytes were differentiated with trophoblast conditioned media (CM; trophoblast-educated macrophages; TEM) for 6 d. Following differentiation cells were treated with LPS (10 ng/ml) in the presence or absence of the TBK inhibitor BX795, after which protein was extracted. **A**. Phosphorylation of IRF3 was determined Western blot analysis in TEMs treated with LPS in the presence or absence of BX795. LPS enhanced pTBK and this effect was blocked by BX795. Representative figure of 3 independent experiments. **B**. LPS treatment increases IFN- $\alpha$  and IFN- $\beta$  mRNA expression in TEMs, which was inhibited in the presence of BX795. Data was presented as fold change of LPS (treated) vs. vehicle (control).  $n = 6$ .  $^{**}P < 0.01$ ; data is presented as mean  $\pm$  SEM



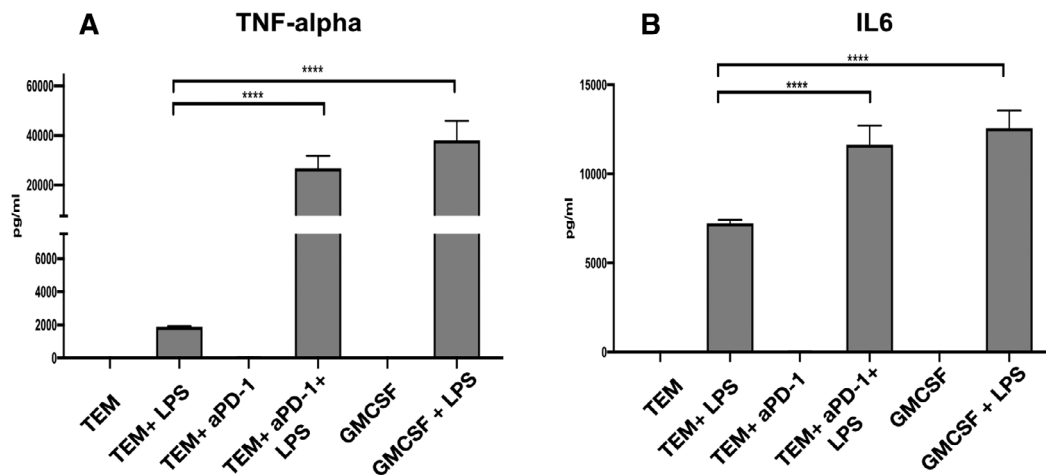
**FIGURE 8** LPS-TLR4 ligation in trophoblast-educated macrophages (TEMs) induce IFN- $\beta$ -related genes via TBK/IRF3 signaling. TEMs were exposed to LPS for 4 h in the presence or absence of the TBK inhibitor BX795, after which RNA was extracted and mRNA expression was determined by qPCR. **A**. LPS treatment increased CCL5 (RANTES) and CXCL10 (IP-10) mRNA expression in TEMs, which was inhibited in the presence of BX795. Data was presented as fold change of LPS (treated) vs. vehicle (control). **B**. LPS treatment increases BD1, BD2, and SLP-1 mRNA expression in TEMs, which is inhibited in the presence of BX795. Data was presented as fold change of LPS (treated) vs. vehicle (control).  $^{*}P < 0.05$ ;  $^{**}P < 0.01$ ;  $^{****}P < 0.001$ ; data is presented as Mean  $\pm$  SEM.  $n = 6$

of PD-L1 but not its membranal form. Therefore, our next objective was to characterize the expression of PD-L1 in trophoblast cells using first trimester trophoblast cell lines Swan 71 and 3A as well as the primary cultures of trophoblast cells isolated from first trimester placentas (obtained from elective terminations). Firstly, we confirmed that trophoblast cells constitutively express PD-L1 by Western blot

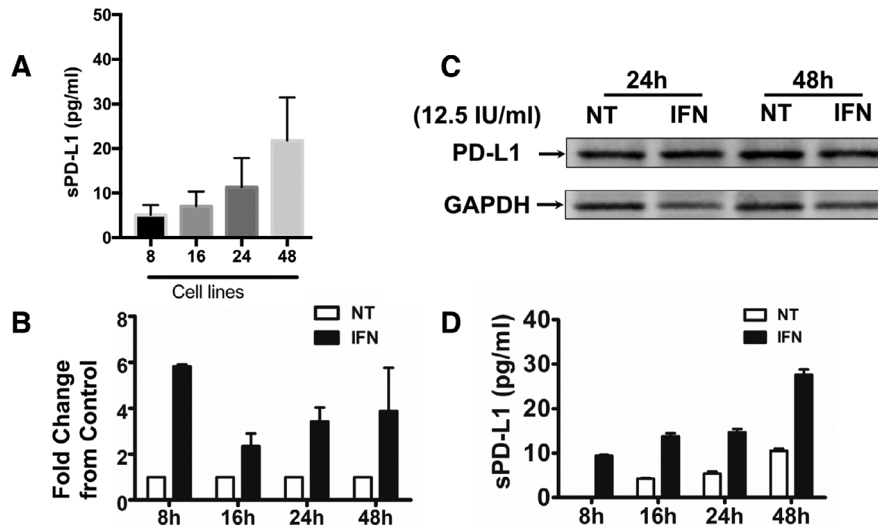
analysis. As shown in Supporting Information Figure S2, positive PD-L1 protein expression was observed in cell lysates from first trimester trophoblast Swan 71 cell line collected at multiple times (Supporting Information Fig. S2). Similar results were observed with 3A and the primary cultures of trophoblast cells (Supporting Information Fig. S3A). Next, we evaluated if PD-L1 was secreted by collecting



**FIGURE 9** Trophoblast derived soluble PD-L1 (sPD-L1) promotes M2 like macrophage polarization. Freshly isolated CD14<sup>+</sup> monocytes undergo differentiation in the presence of GM-CSF (M1 like) or trophoblast conditioned media (CM) in the presence or absence of anti-PD-1 mAb or control isotype for 7 d, after which cells were evaluated by flow cytometry. **A.** Flow cytometry of freshly isolated CD14<sup>+</sup> monocytes differentiated in the presence of trophoblast CM and isotype control. Trophoblast-educated macrophages (TEMs) are CD14<sup>+</sup>/CD206<sup>+</sup>/CD86<sup>-</sup>. **B.** Flow cytometry of freshly isolated CD14<sup>+</sup> monocytes differentiated in the presence of trophoblast CM and PD-1 blocking monoclonal antibody. The presence of the PD-1 blocking mAb prevented trophoblast-induced M2-like polarization, leading to macrophages polarization characterized as increased CD86<sup>+</sup> and decreased CD206<sup>+</sup> cells, similar to the polarization seen with GM-CSF treatment (M1 like). **C.** Flow cytometry of freshly isolated CD14<sup>+</sup> monocytes differentiated in the presence of GM-CSF. GM-CSF induced the polarization of CD14<sup>+</sup> with a relatively high fraction of CD86<sup>+</sup> and a low fraction of CD206<sup>+</sup> macrophages. Representative figure of 3 independent experiments in triplicates



**FIGURE 10** Inhibition of PD-1 changes the cytokine profile of trophoblast-induced macrophage polarization. Freshly isolated CD14<sup>+</sup> monocytes were exposed to TCM in the presence or absence of anti-PD-1 mAb for 7 d, after which cell were exposed to LPS (10 ng/ml) for 24 and TNF- $\beta$  and IL6 cytokine responses were determined by ELLA assay. Note the differential response of trophoblast-educated macrophages (TEMs) when PD1 was blocked during differentiation with a blocking anti-PD-1 mAb. **A.** TEMs are characterized by low expression of TNF- $\alpha$  in response to LPS. However, the presence of anti-PD-1 mAb during differentiation leads to high levels of TNF $\alpha$  expression in response to LPS; **B.** TEMs are characterized by low expression of IL-6 in response to LPS. However, the presence of anti-PD1 mAb during differentiation leads to high levels of IL-6 expression in response to LPS. TEM + LPS = TEMs treated with LPS for 24 h TEM $\alpha$ PD-1 = TEMs differentiated in the presence of  $\alpha$ -PD1 monoclonal antibody. TEM $\alpha$ PD-1 + LPS = TEMs differentiated in the presence of  $\alpha$ -PD-1 monoclonal antibody treated with LPS for 24 h. GMCSF = macrophages differentiated in the presence of GM-CSF. GMCSF + LPS = GM-CSF differentiated macrophages treated with LPS for 24 h. Data presented as mean  $\pm$  SD and are shown for 3 independent experiments with each done in triplicate. \*\*\*\* =  $P < 0.001$



**FIGURE 11** Trophoblast constitutively secrete soluble PD-L1 (sPD-L1), and it is regulated by IFN- $\beta$ . **A**. Expression levels of secreted PD-L1 detected in trophoblast supernatants by ELLA assay. Supernatants were collected from 100% confluent Swan 71 trophoblast cells at different times and PD-L1 expression was quantified by ELLA assay. Note the increase of sPD-L1 in the supernatant in a time-dependent manner. **B**. Trophoblast Swan 71 cells were treated with or without IFN- $\beta$  (12.5 IU/ml) for 8, 16, 24, and 48 h, after which PD-L1 mRNA expression was determined by qPCR. IFN- $\beta$  induced a 6-fold increase in mRNA expression within 8 h of treatment and remained higher than the control for an additional 48 h. Data is presented as fold changes between the IFN- $\beta$  treated group compared to the nontreated control group. **C**. Trophoblast Swan 71 cells were treated with or without IFN- $\beta$  (12.5 IU/ml) for 24 and 48 h, after which PD-L1 protein expression was determined in total cell lysate by Western blot analysis. Trophoblast cells constitutively express intracellular PDL-1 protein, but it is not affected by IFN- $\beta$  treatment. **D**. Trophoblast Swan 71 cells were treated with or without IFN- $\beta$  (12.5 IU/ml) for 24 and 48 h, after which supernatants were collected and sPD-L1 protein expression was determined by ELLA assay. IFN- $\beta$  treatment of trophoblast cells enhances the concentration of sPD-L1 detected in the supernatants in a time-dependent manner.  $n = 6$  individual experiments

culture supernatants from the trophoblast cells at different time points, and sPD-L1 was quantified using the ELLA platform. As shown in Figure 11A, sPD-L1 was detected in the supernatants of trophoblast Swan 71 cells and its concentration increased in a time-dependent manner suggesting constitutive production and secretion of sPD-L1 by trophoblast cells. Similar time-dependent increases of sPD-L1 concentrations were also observed in the supernatants of trophoblast primary cultures (Supporting Information Fig. S3A).

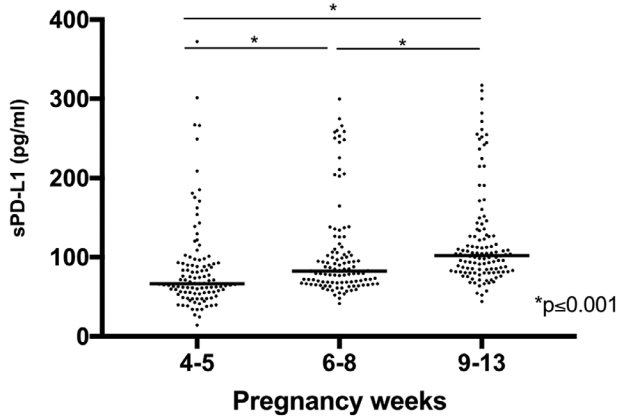
### 3.6 | Stimulation of sPD-L1 secretion by INF- $\beta$ in trophoblast cells

Our next objective was to characterize the regulation of PD-L1 expression and secretion in trophoblast cells by identifying the potential factors that could promote sPD-L1 expression. Because PD-L1 is an IFN-stimulated gene (ISG) and is induced by IFN- $\beta$  in the trophoblast,<sup>51</sup> we tested the hypothesis that a regulatory network would exist wherein IFN- $\beta$  expressed by macrophages could enhance PD-L1 expression in trophoblast cells. To test this hypothesis, we treated trophoblast cells with IFN- $\beta$  (12.5 IU/ml) for 8, 16, 24, and 48 h and evaluated PD-L1 mRNA and protein expression. Interestingly, treatment of trophoblast Swan 71 cells with IFN- $\beta$  induced a time-dependent increase in PD-L1 mRNA (Fig. 11B) but this was not associated with a corresponding increase in the intracellular PD-L1 protein levels (cell lysate) (Fig. 11C). The lack of correlation between the mRNA and intracellular protein levels could be attributed to

either post-transcriptional regulation or continuous secretion of the protein. To test the latter possibility, we collected supernatants from trophoblast cell line Swan 71 following treatment with IFN- $\beta$  (12.5 IU/ml) and quantified the protein concentration of secreted PD-L1. As shown in Figure 11D, protein levels detected in the supernatants of trophoblast Swan 71 cells increased in a time-dependent manner and were further enhanced when trophoblast cells were treated with IFN- $\beta$ . Similar results were found with 3A trophoblast cells (Supporting Information Fig. S3B). The regulation of PD-L1 by IFN- $\beta$  is specific as shown by a dose-dependent increase on PD-L1 mRNA expression observed in Swan 71 cell line as well as in primary cultures of first trimester trophoblast (Supporting Information Fig. S3C and D). These findings show that the trophoblasts constitutively secrete sPD-L1, and its expression and secretion are enhanced by IFN- $\beta$ .

### 3.7 | sPD-L1 expression in serum from pregnant women through early gestation

Finally, we evaluated whether the in vitro observation could be correlated with the in vivo condition during human pregnancy. Thus, we sought to evaluate whether we could detect the presence of circulating sPD-L1 in the serum of pregnant women and the earliest stage of the pregnancy that could be measured. To achieve this objective, we determined the levels of sPD-L1 in serum samples obtained during the first trimester of pregnancy using ELLA assay. Serum samples were categorized into the following groups: peri-implantation phase (4–5 wk),



**FIGURE 12** Determination of circulating soluble PD-L1 (sPD-L1) in the serum of pregnant women during early gestation. Serum samples were collected from pregnant women at gestational weeks 4–13 and sPD-L1 was analyzed by ELLA system. \* $P < 0.001$

histotrophic phase (6–8 wk), and perfusion phase (9–13 wk), based on gestational age at the time of blood draw. These phases are histologically accepted and correlates with the different stages of placentation. As shown in Figure 12, we observed that sPD-L1 could be detected and measured in the serum of pregnant women as early as 4–5 wk. Moreover, through the entire first trimester period, the sPD-L1 circulating concentrations increased as the pregnancy progressed, reaching the highest level at the perfusion phase, the time of establishment of the uteroplacental circulation (Fig. 12).

## 4 | DISCUSSION

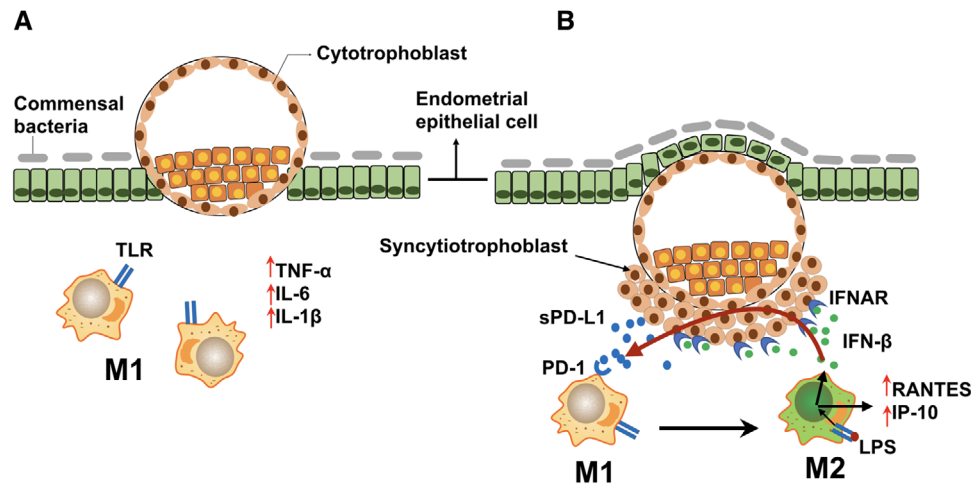
Macrophage polarization in response to the microenvironment is an essential step for maintaining tissue homeostasis, and during pregnancy is essential for its success.<sup>3</sup> In this study, we demonstrate that trophoblasts play a critical role in the process of macrophage polarization, which is mediated, in part, by the expression of sPD-L1. The present study describes, for the first time, the characterization of the TEMs that are differentiated by trophoblast-secreted factors, and demonstrates that the trophoblasts have the capacity to induce, in vitro, the polarization of macrophages into a phenotype that resembles decidual macrophages positive for CD206 and negative for CD86. And in stark contrast to circulating monocytes or M-CSF-differentiated macrophages, TEMs stimulated by TLR4 are characterized by a type I IFN signature.

Macrophages are one of the main leukocyte populations present throughout gestation,<sup>52</sup> characterized by a high degree of plasticity and heterogeneity.<sup>53</sup> As macrophage differentiation is specifically regulated and heavily influenced by their environment, we proposed that the embryo, more specifically the trophoblast cells, might play a role in their differentiation. In order to prove our hypothesis, we developed an in vitro system that could mimic the interaction between these two cell types and showed that trophoblast-secreted factors promoted macrophage differentiation into a unique phenotype that resembled decidual macrophages (CD14<sup>+</sup>/CD16<sup>+</sup>/CD206<sup>+</sup>/CD86<sup>-</sup>).<sup>9,50</sup>

When we evaluated the gene expression profile of these TEMs and compared them with peripheral blood monocytes or M-CSF (M2-like) differentiated macrophages, we observed that TEMs had transcripts such as IFN- $\alpha$  and IFN- $\beta$  indicative of immune regulation and tissue repair. Interestingly, TEM's response to TLR4 stimulation with LPS was associated with the up-regulation of the expression of TRIF/IRF3-dependent genes such as IFN- $\beta$ , CCL5, and CXCL10 as well as the increased expression of CD16. Monocytes/macrophages respond to Gram-negative bacteria through the TLR4 signaling<sup>54,55</sup> and it goes through two distinct pathways depending on the involvement of the adaptor molecules MyD88 or TRIF.<sup>42,54-56</sup> Either pathway leads to distinct outcomes: the MyD88-dependent pathway is characterized by a dominant proinflammatory response (IL-6, TNF- $\alpha$ , CXCL1) through the activation of NF- $\kappa$ B,<sup>57,58</sup> whereas the TRIF-dependent pathway leads to the phosphorylation of TBK and IRF3 and type I IFN expression.<sup>42</sup> In this study, we show that the responses of TLR4 to LPS in macrophages differentiated in the presence of trophoblast-derived factors are mainly through the TRIF/TBK/IRF3-dependent pathway leading to the expression of type I IFN- $\beta$ . Our findings are consistent with the report of decreased expression of inflammatory cytokines in the monocytes/macrophages isolated from pregnant women and challenged by LPS compared to the LPS treated macrophages from nonpregnant women.<sup>52</sup> This differential response was suggested as a mechanism of tolerance to LPS.<sup>52</sup> However, our findings of the activation of the TLR4/TBK/IRF3 pathway and expression of type I IFN suggest that the polarization of decidual macrophages is associated not only with LPS tolerance but also with immune regulation and protection against viral infections, characteristics of M2b macrophages.<sup>59</sup>

Polarized macrophages can be generally classified in two main groups: M1 or classically activated proinflammatory macrophages and M2 or alternatively activated macrophages, which drive immune regulation and tissue remodeling.<sup>60</sup> However, this classification has been shown to be an oversimplification.<sup>61</sup> M2 macrophages can be further subdivided into M2a, M2b, M2c, and M2d based upon the applied stimuli and the resultant transcriptional changes.<sup>62,63</sup> When we compared TEMs with the classical M-CSF differentiated M2 macrophages we observed some similarities but also some major differences between the two types, suggesting that TEMs are potentially a unique subtype of M2b macrophages.

M2b macrophages are known as regulatory macrophages, they can control the depth of the immune response and the inflammatory reaction<sup>62</sup> and promote tissue repair, such as in case of spinal cord injury and myocardial ischemia/reperfusion injury where they have shown to contribute to recovery of these injuries.<sup>64,65</sup> Likewise, decidual macrophages are essential for the continuous tissue renewal associated with trophoblast invasion and placental growth.<sup>66</sup> Comparably, we showed that TEMs had a higher capacity of promoting wound repair than monocytes or M-CSF differentiated macrophages.<sup>14</sup> The induction of IRF3 by TLR4 stimulation further suggests that TEMs more resemble an M2b phenotype. IRF3 has been shown to play a core role in M2b activation<sup>67</sup> and promote the expression of type I IFN- $\beta$ , another characteristic of M2 macrophages.<sup>67</sup>



**FIGURE 13** Regulatory circuit at the maternal fetal interface promoting M2 macrophage polarization. During gestation, macrophages undergo dynamic changes, predominantly displaying the M1 or M2 phenotype. **A.** During the peri-implantation period, activated endometrial/decidua macrophages (M1 phenotype) produce inflammatory cytokines and chemokines (e.g., TNF- $\alpha$ , IL-6, IL-1 $\beta$ ), inducing proinflammatory responses necessary for embryo implantation and trophoblast invasion. **B.** As the trophoblasts invade deeply into the uterine stroma, trophoblast-secreted factors (such as sPD-L1) promote the shift of decidual macrophages into M2 phenotype. LPS/TLR4 ligation induces the expression of IFN- $\beta$ , which further promotes sPD-L1 expression/secretion by trophoblast cells. The up-regulated sPD-L1 enhances macrophage polarization toward an M2b phenotype that consequently decreases inflammation and promotes tissue repair and tolerance.

An additional characteristic of decidual macrophages and TEMs is CD16 expression (CD14<sup>+</sup>/CD16<sup>+</sup>), which may be a potential mechanism by which trophoblast cells modulate TLR4 response to LPS in macrophages. Indeed, Shalova et al.<sup>68</sup> reported that CD16 expression in monocytes contributed to the enhanced expression of the TLR4/TRIF-dependent genes.

What is the functional consequence of trophoblast-induced macrophage polarization and the preferential IFN- $\beta$  response upon TLR stimulation? IFN- $\beta$  is a major mediator of antiviral response.<sup>69,70</sup> In addition to its antiviral role, IFN- $\beta$  has been reported to function as an immune modulator by limiting immune responses<sup>71</sup> and regulating the recruitment of immune cells.<sup>30,72</sup> The immune regulatory functions of IFN- $\beta$  are mediated by the expression of ISGs, which can promote apoptosis,<sup>73</sup> inhibit cell recruitment,<sup>74</sup> and induce cell differentiation.<sup>75</sup> PD-L1 belongs to the ISG family and is an important immune modulatory factor.<sup>76,77</sup>

Recent studies have shown that the PD-1/PD-L1 pathway is critical to the regulation of immune cell homeostasis, especially T cell activation in relation to peripheral tolerance in several malignancies such as cancer as well as during pregnancy to paternal antigens.<sup>78,79</sup> During pregnancy, PD-L1 was shown to be expressed in the placenta of normal pregnancies, primarily in the trophoblasts, and its expression was significantly decreased in pregnancy complications such as recurrent miscarriages.<sup>17,50,79,80</sup> These findings suggest that the placenta can modulate decidual immune cells and that alterations on PD1/PD-L1 axis may affect the crosstalk between the placenta and the maternal immune system.<sup>81</sup>

More recently, we and others have shown a role of the PD-1/PD-L1 axis in macrophage differentiation<sup>3,49,50,80</sup>; and because pregnancy is a dynamic and highly regulated immunologic process, the success of the pregnancy requires that the macrophage activation status remains

appropriately regulated throughout pregnancy. Here, we demonstrate that trophoblast-secreted sPD-L1 may be one of the main mediators of macrophage polarization during pregnancy. Indeed, when we used trophoblast condition media to coculture with monocytes, we showed that macrophages differentiated into CD14<sup>+</sup>/CD206<sup>+</sup>/CD86<sup>-</sup>; however, if we blocked the PD-1 pathway in the monocytes, we were able to inhibit the effect of trophoblast condition media and to shift macrophage differentiation toward CD14<sup>+</sup>/CD206<sup>-</sup>/CD86<sup>+</sup>. Notably, PD-1 blockage enhanced the production of proinflammatory cytokines further confirming the modulatory effects of trophoblast-secreted sPD-L1 on macrophage differentiation.

During early pregnancy, macrophages present at the implantation site possess characteristics associated with M1 phenotype<sup>3</sup>; however, as pregnancy progresses and the developing placenta invades the endometrium and establishes a close contact with decidual cells, the trophoblasts can change the microenvironment and induce the M2 polarization characteristic of decidual macrophages<sup>8</sup> (Fig. 13). Using the *in vitro* model described in this study we are able to recapitulate the trophoblast's function of regulating macrophage polarization. Consequently, we propose that the signals originated from trophoblast cells, by regulating macrophage polarization, are responsible for the shift of the inflammatory milieu at the implantation site (Fig 13).

In line with previous studies,<sup>79,82</sup> we found that both membrane and sPD-L1 constitutively expressed in trophoblast. However, compared with membrane PD-L1 protein, we observed high levels of sPD-L1 in the supernatant of trophoblast cells, which was further enhanced by exposure to IFN- $\beta$ . The presence of a secreted form of PD-L1 observed from the trophoblast could explain the source of the detected circulating sPD-L1 identified in the serum of pregnant women. A previous report showed that circulating PD-L1 protein expression increased from week 14 of gestation until the end of pregnancy.<sup>82,83</sup> However, it



is unknown how early PD-L1 is produced and detectable in the maternal circulation. In this study, we were able to detect sPD-L1 in the serum of pregnant women as early as 4 wk of gestation and its rise throughout the first trimester. To our knowledge, this is the first study to report the presence of circulating sPD-L1 in the serum of normal pregnancies during the first trimester. Therefore, based on these clinical observations and our new findings reported in this study, we postulate that the increase in sPD-L1 in the maternal blood likely indicates the status of placental growth and the immune modulatory function to promote and maintain immune tolerance during gestation.<sup>79</sup> An important component of this immune modulatory function is associated with macrophage polarization (Fig. 13).

## 5 | CONCLUSION

In conclusion, our findings suggest the existence of a regulatory circuit at the maternal-fetal interface wherein IFN- $\beta$  promotes sPD-L1 expression/secretion by trophoblast cells, which then initiates a PD-L1/PD-1-mediated macrophage polarization toward an M2b phenotype, leading to inhibition of inflammation and promotion of tissue repair and tolerance. Our findings have important implications for better understanding of the physiologic mechanisms that control macrophage differentiation, promote maternal-fetal tolerance, and identify potential biomarkers to monitor placental growth and immune modulation throughout gestation.

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## AUTHORSHIP

Y.H.Z. and P.A. performed experiments, interpreted data, and assisted with preparation of the manuscript. Y.Y., J.D., S.G., H.L., and A.H.L. performed the experiments. J.K., J.F.P., E.L., M.J.P., S.S., and L.P. assisted with clinical samples. G.P. performed the statistical analyses. G.M. conceived and designed the study, interpreted data, and wrote the manuscript. Yong-Hong Zhang and Paulomi Aldo are joint first authors.

## DISCLOSURES

The authors declare no conflicts of interest.

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## SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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