MIF IS AMONG THE PROINFLAMMATORY CYTOKINES INCREASED BY LPS IN THE HUMAN TROPHOBLAST LINE

Milica Jovanović-Krivokuća[1], Ivana Stefanoska[1], Tamara Abu Rabi[1], Aleksandra Vilotić[1], Miloš Petronijević[2], Svetlana Vrzić-Petronijević[2], Ljiljana Radojčić[3] and Ljiljana Vićovac[1,*]

[1] *Laboratory for Biology of Reproduction, Institute INEP, University of Belgrade*, Banatska 31b, 11080 Belgrade-Zemun, Serbia

[2] *Medical Faculty, University of Belgrade*, Doktora Subotića 8, 11000 Belgrade, Serbia

[3] *Military Medical Academy*, Crnotravska 17, 11000 Belgrade, Serbia

*Corresponding author: vicovac@inep.co.rs

Abstract: Infection is increasingly considered to contribute to pathological conditions in pregnancy. The placenta acts as a protective immunological fetomaternal barrier which recognizes microbes by pattern recognition receptors on the trophoblast. Lipopolysaccharide (LPS) is a cell wall constituent of Gram-negative bacteria that elicits a strong immune response. In this study, LPS from *E. coli* was used to treat the HTR-8/SVneo trophoblast cell line and examine its influence on cytokines IL-6, IL-8 and MIF using real-time PCR, metalloproteinases (MMP)-2 and -9 by gelatin zymography, and Western analysis of integrin subunits α_1 and β_1, all known to contribute to migration of human trophoblasts *in vitro*. The results described herein for the first time, show that MIF mRNA and secreted MIF protein were significantly elevated (2.5-3- and 2-fold, respectively) in LPS-treated cells. MMP-2 and MMP-9 levels were increased, as well as cell migration, as judged by a wound-healing test, however, no changes in the studied integrin subunits, cell viability or cell numbers were observed. The data obtained furthers our understanding of LPS actions on the trophoblast *in vitro*, additionally implicate MIF, and suggest that infection *in vivo* could indeed alter the functional characteristics of the trophoblast.

Key words: trophoblast; HTR-8/SVneo; cytokines; MIF; LPS

INTRODUCTION

Trophoblast invasion into the uterine stroma and spiral arteries is a crucial step for implantation and the establishment of normal pregnancy [1]. During differentiation along the invasive pathway, extravillous trophoblast cells undergo a change in phenotype [2]. Trophoblast cells invading from the anchoring chorionic villi of the placenta into the uterus lose the adhesion molecules specific for the epithelia and acquire others, such as integrin $\alpha_5\beta_1$ and $\alpha_1\beta_1$ [3]. The process of trophoblast invasion is achieved, among other mechanisms, through degradation of the extracellular matrix by proteolytic enzymes, including matrix metalloproteinases (MMP) -2 and -9 [4,5]. Various autocrine and paracrine factors, including cytokines IL-6, IL-8 and MIF, have been suggested to modulate trophoblast function and control implantation [6-10].

With the establishment of pregnancy there is a marked influx into the uterine endometrium of immune cells that participate in the establishment of pregnancy and immunotolerance of the fetus. In addition to endocrine and transport functions, the placenta acts as a protective maternal-fetal barrier where the trophoblast recognizes microbes through pattern recognition receptors which participate in the innate immune response against infections [11,12]. Infection is considered to contribute to pathological conditions in pregnancy, including abnormal placentation in preeclampsia. The mechanisms linking infection to placental dysfunction are not sufficiently understood. Bacterial lipopolysaccharide (LPS) is a cell wall constituent of most Gram-negative bacteria that elicits strong immune responses in animals. LPS typically consists of a hydrophobic domain known as lipid A (or endotoxin), a core oligosaccharide and a distal polysaccharide (or O-antigen) [13]. LPS is classically

recognized by immune cells via the pattern recognition receptor Toll-like receptor 4 (TLR4). In macrophages, TLR4 activation triggers the biosynthesis of different mediators of inflammation, such as TNF-α and IL1-β [14,15]. In trophoblast cells, a strong expression of TLR 4 has been reported in villous, intermediate and extravillous trophoblasts [16,17]. Both isolated first trimester trophoblast cells and HTR-8 cell line express TLR-2 and TLR-4 [18]. LPS stimulation via TLR-4 led to the activation of extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) [19]. Increased levels of TLR-4 were observed in placental bed extravillous trophoblasts of patients with preeclampsia [20], as well as cytokines such as TNF-α, IL-6 and monocyte chemoattractant protein-1, increased both systemically and locally in the placenta [21,22]. LPS was shown to increase IL-6 and IL-8 by isolated extravillous trophoblasts through the activation of MAPK signaling [23]. Differential inflammatory responses in trophoblast cell models, JEG-3 and BeWo human choriocarcinoma cell lines to LPS were demonstrated [24]. In the immortalized normal trophoblast cell line HTR-8/SVneo, LPS from *Porphyromonas gingivalis* was shown to increase IL-1β and IL-8 [25].

This study was designed to investigate the effect of *E. coli*-derived LPS on proinflammatory cytokine MIF production in the extravillous trophoblast cell line, HTR-8/SVneo, which could influence functional characteristics, such as viability, cell migration and the expression of markers associated with trophoblast invasive properties.

MATERIALS AND METHODS

Cell culture

The HTR-8/SVneo trophoblast cell line was kindly provided by Dr. Charles Graham (Queen's University, Kingston, Ontario, Canada). This cell line was established from human first trimester placenta explant cultures immortalized by SV40 large T antigen [26, 27]. Cells were cultured at 37°C, 5% CO_2, in RPMI 1640 supplemented with 5% FBS with an antibiotic/antimycotic solution (all from Lonza Group Ltd, Basel, Switzerland).

Determination of viable and adherent cell numbers

The viability and adherent cell numbers of HTR-8/SVneo cells were assessed using the MTT test [28] or crystal violet staining, as described previously [29]. HTR-8/SVneo cells were seeded in 96-well plates in 100 μl of medium (2×10^4/well) and allowed to adhere overnight. Cells were rinsed with PBS and incubated for 6 or 24 h with LPS (*Escherichia coli* 055:B5, Sigma-Aldrich Co., Saint Louis, MO, USA) at 0.1, 1 or 10 μg/ml in complete or serum free RPMI. For the MTT test, 100 μl of MTT (1 mg/ml, Sigma) in 10% FBS/PBS was added to each well upon treatment. After incubation for 2 h at 37°C, the medium was replaced by 1-propanol (100 μl/well) and the plates were shaken to ensure complete solubilization of the blue formazan. Absorbance was measured at 540 nm using a microplate reader (LKB, Vienna, Austria). For determination of adherent cell number, the cells were dried and fixed with ice-cold acetone-methanol for 5 min. Then, 50 μl/well of 0.05% crystal violet in 25% methanol was added. After 5 min incubation, the excess of dye was removed by immersing the plates in water and drying at room temperature. The incorporated dye was dissolved in 0.1 M sodium citrate in 50% ethanol at 100 μl/well. Optical density was read at 540 nm. The results are presented as a percentage of control values obtained for untreated cultures. The experiments were carried out three times, n=6.

Cell migration test

The effect of LPS on cell migration was investigated using a cell-wounding test. Cells at 2.5×10^5 per well were plated in 24-well plates in 300 μl of complete medium and incubated at 37°C in 5% CO_2 until confluence. Cells were then scraped off with a sterile pipette tip, rinsed with PBS, and cultured in medium with or without LPS at 0.1, 1 or 10 μg/ml for 24 h. Preselected fields were photographed at zero point and after 24 h. The distances crossed by the cells were measured electronically. Experimental data were expressed as a percentage of control values. The experiments were carried out three times in duplicate.

Gelatin zymography

HTR-8/SVneo cell matrix metalloproteinase gelatino-lytic activity was studied using SDS-PAGE gelatin zymography. Cells were incubated in complete medium in 24-well plates until confluence, rinsed with PBS and treated with LPS at 0.1, 1 or 10 µg/ml in serum-free medium for 24 h. Media were collected and centrifuged at 300xg. Protein concentrations were determined using BCA kit (Pierce). Gelatinase activities in conditioned media were determined as described previously [30]. Samples were separated using 11% SDS-polyacrylamide gels containing 1 mg/ml of gelatin under non-reducing conditions with protein loading of 20 µg per lane. Following electrophoresis, gels were washed twice for 15 min in 2.5% Triton X-100 (v/v) to remove SDS, and incubated overnight in reaction buffer (50 mmol/l Tris–HCl, pH 7, containing 5 mmol/l $CaCl_2$) at 37°C. Gels were stained with Coomassie brilliant blue G-250 for 30 min at RT and destained in 30% methanol and 10% glacial acetic acid (v/v). Proteinase activity was observed as a clear band of digested gelatin at the designated molecular mass. Gelatinase levels were semi-quantitated by densitometric analysis using the ImageMasterTotalLabv 2.01 program (Amersham Biosciences). Experiments were carried out four times in duplicate.

SDS-PAGE and immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5% polyacrylamide gel for integrin α_1, 10% gel for integrin β_1 and 12.5% gel for MIF. Untreated or LPS-treated HTR-8/SVneo cells were lysed in the sample buffer (0.125 M Tris-HCl containing 4% SDS, 20% glycerol, 0.1% bromophenol blue, 10% 2-mercaptoethanol and protease inhibitor cocktail, Sigma). For secreted MIF, cell conditioned media were mixed 3:1 with sample buffer. Samples were boiled for 5 min. Undissolved material was removed by centrifugation (17000xg for 10 min). Equal amounts of total protein (40 µg for lysates and 10 µg for conditioned media) were loaded per lane. For Western blotting, non-specific binding was blocked with 1% casein, membranes were incubated with mouse anti-MIF (0.5 µg/ml, R&D Systems), mouse anti-integrin α_1 (0.33 µg/ml, R&D Systems) or rabbit anti-integrin β_1 (0.3 µg/ml, R&D Systems) antibody overnight at 4˚C with constant shaking. Biotinylated anti-rabbit

IgG (0.3 µg/ml) or anti-mouse IgG (0.75 µg/ml) secondary antibodies were used, followed by incubation with avidin-biotinylated peroxidase complex (ABC, Vector). The reaction was visualized using a chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA). Staining for actin was used as loading control. Membranes were scanned on an HP Scanjet G3110 and analyzed by the ImageMasterTotalLab v2.01 program (Amersham Biosciences, NJ, USA). Experiments were carried out four times in duplicate.

Real-time PCR

Total RNA was collected from subconfluent cultures of HTR-8/SVneo cells (control or LPS-treated) using TRIzol (Applied Biosystems, Carlsbad, CA, USA), as suggested by the manufacturer. First-strand cDNA was synthesized from 1 µg of total RNA, using 0.2 µg of random hexamer primers, 250 µM of each dNTP and 200U of RevertAid reverse transcriptase (Fermentas, Vilnius, Lithuania). Real-time PCR was performed in a 7500 Real Time PCR System (Applied Biosystems, Carlsberg, USA). The reaction mixture contained 100 ng of cDNA, 5 µl 2x SYBR® Green PCR Master Mix (Applied Biosystems, Carlsberg, USA) and a specific primer in a final concentration of 0.5 µM. Specific primers with the following sequences were used - MIF F: CCGGACAGGGTCTACATCA, MIF R: ATTTCTCCCCACCAGAAGGT; IL-6 F: GAGAAAGGAGACATGTAACAAGAGT, IL-6 R: CGCAGAATGAGATGAGTTGT; IL-8 F: CTCTCTTGGCAGCCTTCCTGATT; IL-8 R: AACTTCTCCACAACCCTCTGCAC; TBP F: GAGCTGTGATGTGAAGTTTCC, TBP R: TCTGGGTTTGATCATTCTGTAG (as an internal control). Melting curve analysis was performed to verify amplification specificity. TBP was used as the endogenous control gene. Calculations were made using the comparative ΔΔCt method [31]. Tree experiments, each in triplicate, were performed.

Statistical analysis

The data were analyzed by Statistical Software Program, version 5.0 (Primer of Biostatistic, McGraw-Hill Companies, Inc., New York, NY, USA) using the non-parametric Mann-Whitney rank sum test and Student's t-test as appropriate. Values were considered significantly different when $p < 0.05$.

RESULTS

Effect of LPS on HTR-8/SVneo cytokine expression

Treatment with LPS induced an increase in IL-6, IL-8 and MIF expression, as determined by the ΔΔCt method (Fig. 1). The previously reported increase in IL-6 and IL-8 RNA levels by LPS was confirmed here. LPS at 0.1, 1 and 10 μg/ml stimulated IL-6 mRNA 2.3-, 3.6- and 3-fold (Fig. 1A), and IL-8 mRNA 2.3-, 1.7-, and 2.1-fold, respectively (Fig.1B). An increased expression of MIF by trophoblasts exposed to LPS, however, was not previously reported. The MIF RNA level was increased 2.3-, 2.8- and 2.5-fold by LPS at 0.1, 1 and 10 μg/ml, respectively (Fig.1C). Secreted MIF protein was also increased in culture media by LPS at 1 μg/ml to 200% of control (Fig.1D).

### Effect of LPS on HTR-8/SVneo cell migration and expression of MMPs and integrin subunits

Since each of the increased cytokines stimulates trophoblast migration, a wound-healing assay was used

to determine whether *E. coli* LPS influenced HTR-8/SVneo cell migration. A moderate increase was observed to 123% of control for 0.1 μg/ml, to 129% of control for 1 μg/ml and to 118% of control for 10 μg/ml (Fig. 2).

In this study, two integrin subunits and MMPs considered particularly relevant effectors for trophoblast migration/invasion were tested. Semi-quantitative analysis by SDS-PAGE gelatin zymography revealed that LPS stimulated MMP-2 levels to 146%, 166% and 154% of control, and MMP-9 to 141%, 160% and 145% of control (Fig. 3A) by LPS at 0.1, 1 and 10 μg/ml, respectively (Fig. 3B). LPS did not change integrins α_1 and β_1 assessed by Western blot (Fig. 3C, D).

Influence of LPS on HTR-8/SVneo cell proliferation

The possibility that LPS may impact HTR-8/SVneo cell viability and adherent cell numbers was tested by MTT and crystal violet staining. None of the concentrations (0.1, 1 and 10 μg/ml) influenced HTR-8/SVneo adherent cell number after 6 h (Fig. 4A) or
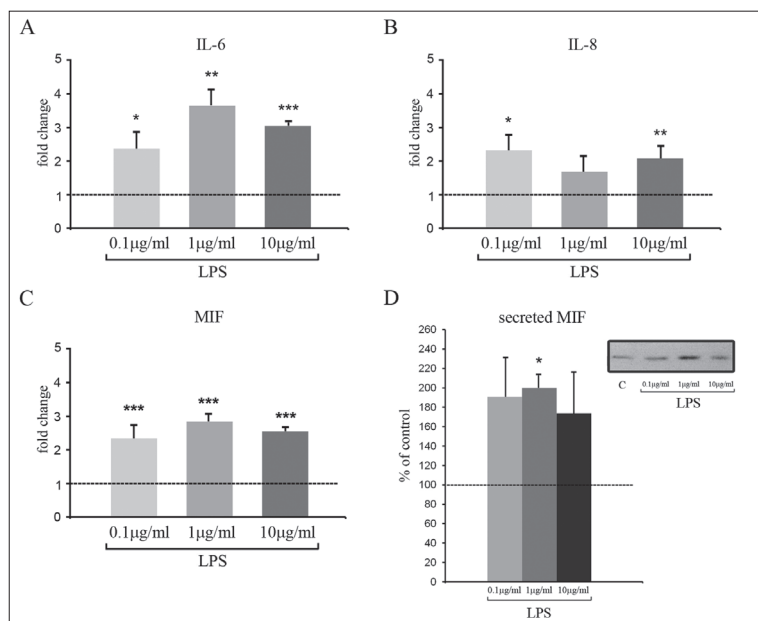
Fig.1. LPS impact on proinflammatory cytokines in HTR-8/SVneo cells. The effect of LPS on the expression of IL-6 (A), IL-8 (B) and MIF (C) as assessed by qPCR. The data are expressed as fold of change±SEM from the untreated control. D. Western blot analysis of the effect of LPS on secreted MIF in HTR-8/SVneo cell culture media. Results of densitometric analysis of the corresponding bands are shown in the chart. The data are expressed as percent of the untreated control±SEM. * p<0.05; ** p<0.01; *** p<0.001.
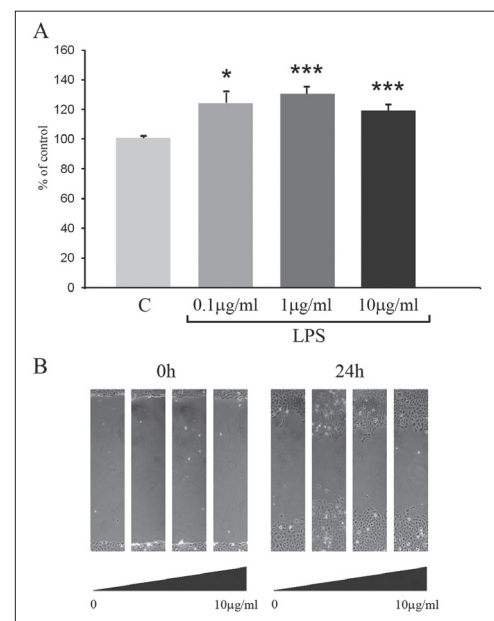
Fig. 2. The effect of LPS on HTR-8/SVneo migration. The distances crossed by cells over a period of 24 h were determined electronically and are given in the chart (A) as percentages of the untreated control±SEM (A). * p<0.05; *** p<0.001. The representative experiment is shown in B.

24 h of culture (Fig. 4C) in serum-free, or complete medium after 24 h incubation (Fig. 4E). No change in cell viability by MTT test was observed under the conditions studied (Fig. 4B, D, F). Therefore, none of the observed changes were induced by altered cell numbers or viability.

DISCUSSION

In human pregnancy, infection and inflammation at term can cause premature rupture of the membranes and delivery [32]. In early pregnancy, infection has been particularly studied with regard to the involvement of

diverse immune cells of the placental bed; however, there is still insufficient knowledge of its effect on first trimester of pregnancy trophoblast function.

LPS impact on trophoblast cell survival has been previously reported as proapoptotic [33,34] or absent [18, 35]. In this study, neither HTR-8/SVneo cell viability nor adherent cell numbers were changed with LPS from *E. coli*, which confirms previous results on HTR-8 cells [18] and other cell types, such as Wharton's jelly-derived mesenchymal stem cells and hepatocytes [36,37].

Various proinflammatory cytokines have been reported to influence trophoblast cell migration and invasion, some of which having an inhibitory ef-
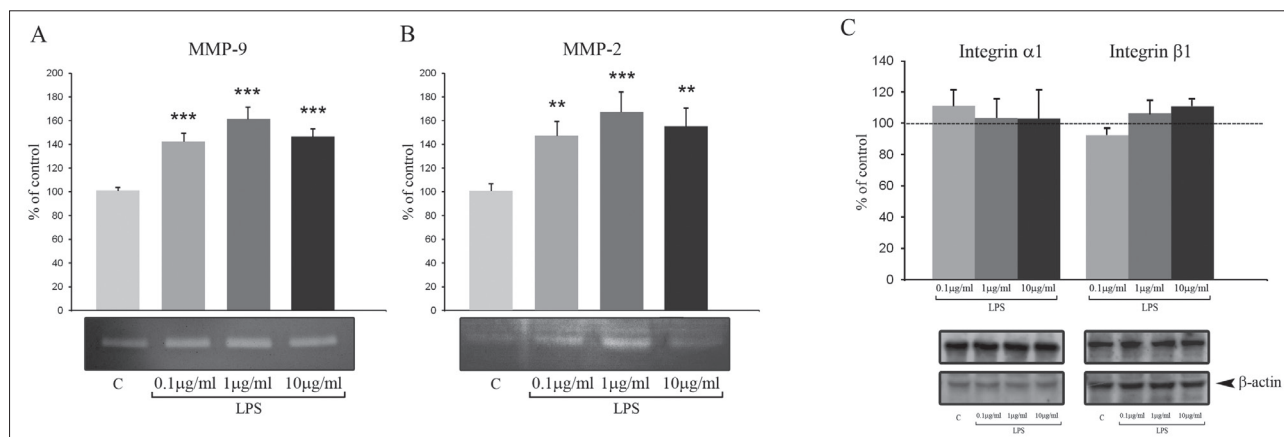
Fig. 3. The effect of LPS on the markers of trophoblast invasive phenotype in HTR-8/SVneo cells. MMP-2 and -9 (A, B), and integrin subunits α_1 and β_1 (C) in LPS-treated HTR-8/SVneo cells, using culture media gelatin zymography and cell lysate Western blot analysis respectively. Results of densitometric analysis of the corresponding bands for MMP-9 (A) and MMP-2 (B) and the integrins α_1 and β_1 (C) are shown in the charts. The data are expressed as percent of the untreated control±SEM (D). ** $p<0.01$; *** $p<0.001$.
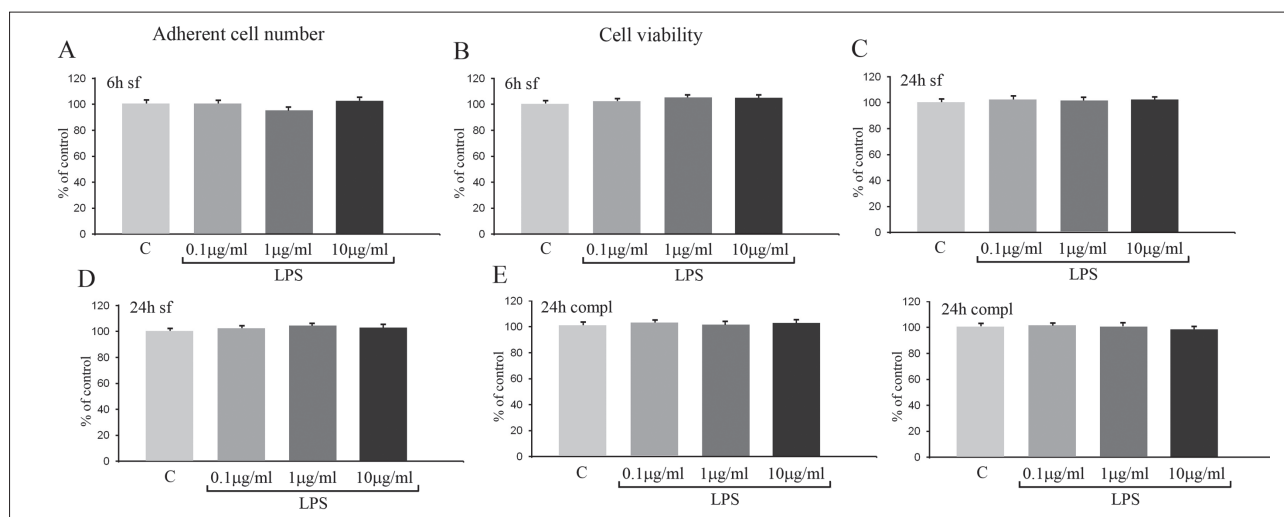
Fig. 4. The effect of LPS on HTR-8/SVneo cell viability and adherent cell number. The HTR-8/SVneo adherent cell number was assessed by crystal violet staining (A, C, D) and cell viability by MTT test (B, D, F) in serum free medium (A, B, C, D) or complete medium (E, F). The data are expressed as percent of the untreated control±SEM.

fect, such as TNF α [38] and IFN γ [39]. Stimulatory cytokines include IL-1β [40], IL-6 [6], IL-8 [41, 7] and recently reported MIF [10]. LPS was shown to increase several inflammatory cytokines in the trophoblast, including IL-1β [42], IL-6 [33,23], IL-8 [43] and RANTES [44]. In this study, the stimulatory effect of LPS on IL-6 and IL-8 expression by HTR-8/SVneo cells was confirmed. The data obtained introduce the important regulator of innate immunity, MIF, to the list of LPS-induced cytokines in the human trophoblast. Systemic MIF increase has been implicated in infection and severe sepsis [45]. Specifically, MIF was identified as a major secreted protein released by anterior pituitary cells in response to LPS stimulation [46], contributing to circulating MIF present in the post-acute phase of endotoxemia. The cytokines increased with LPS, IL-6, IL-8 and MIF, could be assumed to act in an autocrine fashion to stimulate HTR-8/SVneo migration *in vitro.* Indeed, an increase in HTR-8/SVneo cell migration was observed in this study in cultures treated with LPS. In previous studies, each of these cytokines was shown to increase cell migration when added individually to cell culture media [6,7,10]. Interestingly, two commonly used trophoblast cell models, choriocarcinoma cell lines JEG-3 and BeWo, were shown to respond differently to LPS [24]. In JEG-3 cells, a dose- and time-dependent stimulation of IL-6 and TNF-α and increased NF-κB gene expression were observed, which was not replicated in BeWo cells.

Trophoblast migration and invasion are prerequisites for the establishment of a healthy pregnancy. Our finding that *E. coli* LPS induced a small but significant stimulatory effect on HTR-8/SVneo cell migration differs from the previous study [23], where extravillous trophoblast invasion was inhibited. In addition to the intrinsic discrepancy between these two test models, the obtained result could be due to different LPS exposure times (24 h here as opposed to 72 h in the previous study). LPS from *Porphyromonas gingivalis* had no effect on HTR-8/SVneo migration, but when coadministered with nicotine cell migration was inhibited [47]. Nevertheless, LPS stimulated the migration and/or invasion of other cell types such as dendritic cells [48], colorectal cancer cells [49] and vascular smooth muscle cells [50], while it has been reported to have a dose-dependent differential effect on human dental pulp stem cells [51].

Trophoblast cell migration and invasion are known to depend on integrin levels and MMP secretion and activity [52]. MMP-2 and -9, as significant mediators of trophoblast invasion, were stimulated here by LPS. This is in accordance with previous studies on the trophoblast where MMP-2 [33] and MMP-9 [53] were also stimulated. LPS had a similar effect in other cell types as well, increasing these MMPs in dental pulp [54], osteosarcoma [55] and colorectal cancer cells [49]. No information regarding the modulation of integrins by LPS in trophoblast cells is available. The integrin subunits most relevant for trophoblast migration/invasion, α_1 and β_1, were investigated here and found to be unaffected by LPS from *E. coli*.

Based on our results and previous reports, LPS could have a differential effect on trophoblast migration/invasion *in vitro*, depending on the concentration, type of LPS and the exposure time. *In vivo,* the situation is far more complex, since LPS affects multiple immune and non-immune cell types present in the placental bed. For instance, LPS activated macrophages and their products could modulate direct LPS effects on the trophoblast, as observed *in vitro* [56]. On the other hand, trophoblast cells were shown to secrete chemokines able to recruit maternal macrophages and alter their cytokine profile [57], and also to modulate activation profile and migration of monocytes *in vitro*, which was restricted in the presence of LPS [58]. In addition to extravillous trophoblasts, syncytiotrophoblasts were also shown to respond to LPS by a large increase in IL-8 [59]. Thus, multiple signaling circuits are likely affected in infection with Gram-negative bacteria, and our finding that MIF (a potent proinflammatory cytokine) is also stimulated in the trophoblast by LPS adds yet another element to this complexity.

Authors' contributions: MJK, TAR, IS and AV performed the experiments. MP, LR, SVP, IS, AV and TAR analyzed the data and edited the text. MJK and LV conceived and designed the study, analyzed the data and wrote the paper.

Conflict of interest disclosure: The authors declare no conflict of interest.

REFERENCES

1. Pijnenborg R, Bland JM, Robertson WB, Brosens I. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. Placenta. 1983;4(4):397-413.

2. Vićovac L, Aplin JD. Epithelial-mesenchymal transition during trophoblast differentiation. Acta Anat (Basel). 1996;156(3):202-16.

3. Damsky CH, Fitzgerald ML, Fisher SJ. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. J Clin Invest. 1992;89(1):210-22.

4. Lala PK, Graham CH. Mechanisms of trophoblast invasiveness and their control: the role of proteases and protease inhibitors. Cancer Metastasis Rev. 1990;9(4):369-79.

5. Librach CL, Werb Z, Fitzgerald ML, Chiu K, Corwin NM, Esteves RA, Grobelny D, Galardy R, Damsky CH, Fisher SJ. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J Cell Biol. 1991;13(2):437-49.

6. Jovanović M, Vićovac L. Interleukin-6 stimulates cell migration, invasion and integrin expression in HTR-8/SVneo cell line. Placenta. 2009;30(4):320-8.

7. Jovanović M, Stefanoska I, Radojcić L, Vićovac L. Interleukin-8 (CXCL8) stimulates trophoblast cell migration and invasion by increasing levels of matrix metalloproteinase (MMP)2 and MMP9 and integrins alpha5 and beta1. Reproduction. 2010;139(4):789-98.

8. Soares MJ, Chakraborty D, Kubota K, Renaud SJ, Rumi MA. Adaptive mechanisms controlling uterine spiral artery remodeling during the establishment of pregnancy. Int J Dev Biol. 2014;58(2-4):247-59.

9. Fritz R, Jain C, Armant DR. Cell signaling in trophoblast-uterine communication. Int J Dev Biol. 2014;58(2-4):261-71.

10. Jovanović Krivokuća M, Stefanoska I, Abu Rabi T, Al-Abed Y, Stošić-Grujičić S, Vićovac Lj. Pharmacological inhibition of MIF interferes with trophoblast cell migration and invasiveness. Placenta. 2015;36(2):150-9.

11. Guleria I, Pollard JW. The trophoblast is a component of the innate immune system during pregnancy. Nat Med. 2000;6(5):589-93.

12. Abrahams VM, Mor G. Toll-like receptors and their role in the trophoblast. Placenta. 2005;26(7):540-7.

13. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem. 2002;71:635-700.

14. Beutler B, Cerami A. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Annu Rev Biochem. 1988;57:505-18.

15. Dinarello CA. Interleukin-1 and interleukin-1 antagonism. Blood. 1991;77(8):1627-52.

16. Holmlund U, Cebers G, Dahlfors AR, Sandstedt B, Bremme K, Ekström ES, Scheynius A. Expression and regulation of the pattern recognition receptors Toll-like receptor-2 and Toll-like receptor-4 in the human placenta. Immunology. 2002;107(1):145-51.

17. Ma Y, Krikun G, Abrahams VM, Mor G, Guller S. Cell type-specific expression and function of toll-like receptors 2 and 4 in human placenta: implications in fetal infection. Placenta. 2007;28(10):1024-31.

18. Abrahams VM, Bole-Aldo P, Kim YM, Straszewski-Chavez SL, Chaiworapongsa T, Romero R, Mor G. Divergent trophoblast responses to bacterial products mediated by TLRs. J Immunol. 2004;173(7):4286-96.

19. Klaffenbach D, Rascher W, Röllinghoff M, Dötsch J, Meissner U, Schnare M. Regulation and signal transduction of toll-like receptors in human choriocarcinoma cell lines. Am J Reprod Immunol. 2005;53(2):77-84.

20. Kim YM, Romero R, Oh SY, Kim CJ, Kilburn BA, Armant DR, Nien JK, Gomez R, Mazor M, Saito S, Abrahams VM, Mor G. Toll-like receptor 4: a potential link between "danger signals," the innate immune system, and preeclampsia? Am J Obstet Gynecol. 2005;193(3 Pt 2):921-7.

21. LaMarca BD, Ryan MJ, Gilbert JS, Murphy SR, Granger JP. Inflammatory cytokines in the pathophysiology of hypertension during preeclampsia. Curr Hypertens Rep. 2007;9(6):480-5.

22. Szarka A, Rigó J Jr, Lázár L, Beko G, Molvarec A. Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and preeclampsia determined by multiplex suspension array. BMC Immunol. 2010 Dec 2;11:59.

23. Anton L, Brown AG, Parry S, Elovitz MA. Lipopolysaccharide induces cytokine production and decreases extravillous trophoblast invasion through a mitogen-activated protein kinase-mediated pathway: possible mechanisms of first trimester placental dysfunction. Hum Reprod. 2012;27(1):61-72.

24. Koh YQ, Chan HW, Nitert MD, Vaswani K, Mitchell MD, Rice GE. Differential response to lipopolysaccharide by JEG-3 and BeWo human choriocarcinoma cell lines. Eur J Obstet Gynecol Reprod Biol. 2014;175:129-33.

25. Riewe SD, Mans JJ, Hirano T, Katz J, Shiverick KT, Brown TA, Lamont RJ. Human trophoblast responses to Porphyromonas gingivalis infection. Mol Oral Microbiol. 2010;25(4):252-9.

26. Graham CH, Hawley TS, Hawley RG, MacDougall JR, Kerbel RS, Khoo N, Lala PK. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. Exp Cell Res. 1993;206(2):204-11.

27. Irving JA, Lysiak JJ, Graham CH, Hearn S, Han VK, Lala PK. Characteristics of trophoblast cells migrating from first trimester chorionic villus explants and propagated in culture. Placenta. 1995 Jul;16(5):413-33.

28. Hanisch FG., Dressen F, Uhlenbruck G. Quantitative microadhesion assay on polystyrene matrices. In: Gabius HJ, Gabius S, editors. Lectins and Glycobiology. Berlin: Springer-Verlag, Heidelberg; 1993. p. 411–7.

29. Stefanoska I, Jovanović Krivokuća M, Vasilijić S, Ćujić D, Vićovac L. Prolactin stimulates cell migration and invasion by human trophoblast in vitro. Placenta. 2013;34(9):775-83.

30. Lash GE, Otun HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Inhibition of trophoblast cell invasion by TGFB1, 2, and 3 is associated with a decrease in active proteases. Biol Reprod. 2005;73(2):374-81.

31. Livak KJ, Schmittgen TD. (). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. Methods. 2001;25: 402-8.

32. Romero R, Roslansky P, Oyarzun E, Wan M, Emamian M, Novitsky TJ, Gould MJ, Hobbins JC. Labor and infection. II. Bacterial endotoxin in amniotic fluid and its relationship to the onset of preterm labor. Am J Obstet Gynecol. 1988;158(5):1044-9.

33. Nakatsuka M, Asagiri K, Noguchi S, Habara T, Kudo T. Nafamostat mesilate, a serine protease inhibitor, suppresses lipopolysaccharide-induced nitric oxide synthesis and apoptosis in cultured human trophoblasts. Life Sci. 2000;67(10):1243-50.

34. Li Y, Shibata Y, Zhang L, Kuboyama N, Abiko Y. Periodontal pathogen Aggregatibacter actinomycetemcomitans LPS induces mitochondria-dependent-apoptosis in human placental trophoblasts. Placenta. 2011;32(1):11-9.

35. Okada T, Matsuzaki N, Sawai K, Nobunaga T, Shimoya K, Suzuki K, Taniguchi N, Saji F, Murata Y. Chorioamnionitis reduces placental endocrine functions: the role of bacterial lipopolysaccharide and superoxide anion. J Endocrinol. 1997;155(3):401-10.

36. Mei YB, Zhou WQ, Zhang XY, Wei XJ, Feng ZC. Lipopolysaccharides shapes the human Wharton's jelly-derived mesenchymal stem cells in vitro. Cell Physiol Biochem. 2013;32(2):390-401.

37. Liu Q, Qian Y, Chen F, Chen X, Chen Z, Zheng M. EGCG attenuates pro-inflammatory cytokines and chemokines production in LPS-stimulated L02 hepatocyte. Acta Biochim Biophys Sin (Shanghai). 2014;46(1):31-9.

38. Bauer S, Pollheimer J, Hartmann J, Husslein P, Aplin JD, Knöfler M. Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures. J Clin Endocrinol Metab. 2004;89(2):812-22.

39. Lash GE, Otun HA, Innes BA, Kirkley M, De Oliveira L, Searle RF, Robson SC, Bulmer JN. Interferon-gamma inhibits extravillous trophoblast cell invasion by a mechanism that involves both changes in apoptosis and protease levels. FASEB J.2006;20(14):2512-8.

40. Librach CL, Feigenbaum SL, Bass KE, Cui TY, Verastas N, Sadovsky Y, Quigley JP, French DL, Fisher SJ. Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. J Biol Chem. 1994;269(25):17125-31.

41. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, Prus D, Cohen-Daniel L, Arnon TI, Manaster I, Gazit R, Yutkin V, Benharroch D, Porgador A, Keshet E, Yagel S, Mandelboim O. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. Nat Med. 2006;12(9):1065-74.

42. Pontillo A, Girardelli M, Agostinis C, Masat E, Bulla R, Crovella S. Bacterial LPS differently modulates inflammasome gene expression and IL-1β secretion in trophoblast cells, decidual stromal cells, and decidual endothelial cells. Reprod Sci. 2013;20(5):563-6.

43. Shimoya K, Moriyama A, Matsuzaki N, Ogata I, Koyama M, Azuma C, Saji F, Murata Y. Human placental cells show enhanced production of interleukin (IL)-8 in response to lipopolysaccharide (LPS), IL-1 and tumour necrosis factor (TNF)-alpha, but not to IL-6. Mol Hum Reprod. 1999;5(9):885.

44. Svinarich DM, Bitonti OM, Araneda H, Romero R, Gonik B. Induction and posttranslational expression of G-CSF and RANTES in a first trimester trophoblast cell line by lipopolysaccharide. Am J Reprod Immunol. 1996;36(5):256-9.

45. Calandra T, Echtenacher B, Roy DL, Pugin J, Metz CN, Hültner L, Heumann D, Männel D, Bucala R, Glauser MP. Protection from septic shock by neutralization of macrophage migration inhibitory factor. Nat Med. 2000;6(2):164-70.

46. Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, Manogue KR, Cerami A, Bucala R. MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature. 1993;365(6448):756-9.

47. Komine-Aizawa S, Hirohata N, Aizawa S, Abiko Y, Hayakawa S. Porphyromonas gingivalis lipopolysaccharide inhibits trophoblast invasion in the presence of nicotine. Placenta. 2015;36(1):27-33.

48. Gröbner S, Lukowski R, Autenrieth IB, Ruth P. Lipopolysaccharide induces cell volume increase and migration of dendritic cells. Microbiol Immunol. 2014;58(1):61-7.

49. Zhang D, Li YH, Mi M, Jiang FL, Yue ZG, Sun Y, Fan L, Meng J, Zhang X, Liu L, Mei QB. Modified apple polysaccharides suppress the migration and invasion of colorectal cancer cells induced by lipopolysaccharide. Nutr Res. 2013;33(10):839-48.

50. Yang G, Zhou X, Chen T, Deng Y, Yu D, Pan S, Song Y. Hydroxysafflor yellow A inhibits lipopolysaccharide-induced proliferation and migration of vascular smooth muscle cells via Toll-like receptor-4 pathway. Int J Clin Exp Med. 2015;8(4):5295-302.

51. Li D, Fu L, Zhang Y, Yu Q, Ma F, Wang Z, Luo Z, Zhou Z, Cooper PR, He W. The effects of LPS on adhesion and migration of human dental pulp stem cells in vitro. J Dent. 2014;42(10):1327-34.

52. Murray MJ, Lessey BA. Embryo implantation and tumor metastasis: common pathways of invasion and angiogenesis. Semin Reprod Endocrinol. 1999;17(3):275-90.

53. Li W, Unlugedik E, Bocking AD, Challis JR. The role of prostaglandins in the mechanism of lipopolysaccharide-induced proMMP9 secretion from human placenta and fetal membrane cells. Biol Reprod. 2007;76(4):654-9.

54. Shin MR, Kang SK, Kim YS, Lee SY, Hong SC, Kim EC. TNF-α and LPS activate angiogenesis via VEGF and SIRT1 signalling in human dental pulp cells. Int Endod J. 2015;48(7):705-16.

55. Roomi MW, Kalinovsky T, Rath M, Niedzwiecki A. In vitro modulation of MMP-2 and MMP-9 in pediatric human sarcoma cell lines by cytokines, inducers and inhibitors. Int J Oncol. 2014;44(1):27-34.

56. Renaud SJ, Macdonald-Goodfellow SK, Graham CH. Coordinated regulation of human trophoblast invasiveness by macrophages and interleukin 10. Biol Reprod. 2007;76(3):448-54.

57. Fest S, Aldo PB, Abrahams VM, Visintin I, Alvero A, Chen R, Chavez SL, Romero R, Mor G. Trophoblast-macrophage interactions: a regulatory network for the protection of pregnancy. Am J Reprod Immunol. 2007;57(1):55-66.

58. Grasso E, Paparini D, Hauk V, Salamone G, Leiros CP, Ramhorst R. Differential migration and activation profile of monocytes after trophoblast interaction. PLoS One. 2014 May 21;9(5):e97147. Available from: http://www.plosone.org/article/citationList.action?articleURI=info%3Adoi%2F10.1371/journal.pone.0097147

59. Ma Y, Kadner SS, Guller S. Differential effects of lipopolysaccharide and thrombin on interleukin-8 expression in syncytiotrophoblasts and endothelial cells: implications for fetal survival. Ann N Y Acad Sci. 2004;1034:236-44.