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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Altered expression of Notch signaling, Tlr receptors, and surfactant protein expression after prostaglandin inhibition may be associated with the delayed labor in LPS-induced mice

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Abstract

Purpose This study aims to investigate whether indomethacin (IND) delays preterm birth by regulating the Notch pathway, Tlr receptors, and Sp-A in the placenta in lipopolysaccharide (LPS)-induced preterm labor (PTL) model.

Methods CD-1 mice were distributed to the pregnant control (PC), Sham, PBS, IND (2 mg/kg; i.p.), LPS (25 µg/100 µl; intrauterine), and LPS + IND groups. The injections were performed on day 14.5 of pregnancy. Placentae were collected on day 15.5 of pregnancy, and immunohistochemical analyzes were performed. Differences in staining intensities between the Cox-1, Notch-1 (N1), Dll-1, Jagged-2 (Jag-2), Tlr-2, and Tlr-4 proteins were compared.

Results Preterm labor rates were 100% and 66% (preterm delivery delayed 5 h) in the LPS and LPS + IND groups, respectively. In LPS-treated mice, a general morphological deterioration was observed in the placenta. Total placental mid-sagittal measurement was significantly reduced in the LPS-treated group, while it was similar to the PC group in the LPS + IND group. Cox-1 expression in the LZ increased, and Sp-A expression decreased after LPS injection, and IND administration diminished this increase. N1 expression increased in the labyrinth zone (LZ) and the junctional zone (JZ). Dll-1 and Jag-2 expression increased in the JZ after LPS injection ($p < 0.0001$). IND administration diminished Tlr-2 expression in the LZ and Tlr-4 expression in the JZ after LPS injection.

Conclusion In conclusion, PG (prostaglandin) inhibition may alter Notch signaling, Tlr, and Sp-A protein expression and may be associated with delayed labor in LPS-induced mice.

Keywords Preterm birth · Notch signaling · Prostaglandin inhibitors · Placenta · Inflammation

Introduction

The placenta, which begins to develop in the early stage of embryogenesis, continues to determine fetal growth until after birth [1]. The placenta mediates the exchange of nutrients and waste at the maternal and fetal interface and provides homeostasis at this highly vascular interface [2]. Although the placenta plays a crucial role in managing the pregnancy process, much remains to be learned about this transient organ [3].

The mature mouse placenta consists of four distinct layers: decidua, spongiotrophoblast (junctional) zone, labyrinth zone, and chorionic plate. The decidua and junctional zones are separated by parietal trophoblast giant cells (TGCs) [4]. In mid-pregnancy, the mouse placenta produces blood cells, and the LZ promotes differentiation of fetal erythrocytes [5]. The LZ is the placental layer, where the maternal-fetal exchange occurs in the trophoblast cell islands. Maternal

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blood vessels contain nucleated erythrocytes, and the endothelium of these vessels is lined with trophoblast cells. Fetal labyrinth vessels have nucleated erythrocytes that are larger than maternal erythrocytes.

On the other hand, the JZ contains fetal erythrocytes at different stages of their development, suggesting erythropoietic differentiation [6]. Between embryonic days 10 (E10) and E14.5, the LZ becomes more complex. The syncytiotrophoblast, formed by the fusion of chorionic trophoblast cells, includes the exchange surface of the labyrinth zone. Trophoblast giant cells are the first to differentiate during embryogenesis and are vital for placentation. Trophoblast giant cells regulate uterine decidualization in early pregnancy and anastomose with maternal blood spaces to form the temporary placenta. Later in pregnancy, TGCs secrete various hormones and paracrine factors to induce vascular remodeling [7]. Various TGC subtypes with different cell origins are found within the mature placenta, including the parietal (P)-TGCs, spiral artery associated (Sp-A)-TGCs, the canal (C)-TGCs, sinusoidal (S)-TGCs, and glycogen (Gly)-TGCs [8]. Spongiotrophoblast (SpT) cells and trophoblast glycogen cells (GCs) make up the majority of the endocrine component of the mouse placenta [9]. As the placenta matures, glycogen-positive cells are observed to form tightly packed oval cells within the JZ [10].

The development of a functional mouse placenta is regulated by many cell-signaling pathways. Notch-1 (N1) is expressed in the JZ [11], and the Notch signaling pathway is thought to be particularly essential for trophoblast vessel invasion [12]. Members of the evolutionarily conserved Notch signaling pathway play a regulatory role at multiple stages, including trophoblast cell differentiation, chorion branching, and fetal vessel formation in the placenta [13]. Except for Dll-3, Notch signaling proteins are also expressed in the human placenta [14]. Notch signaling also has a role in early erythropoiesis [15]. There are four Notch receptors (Notch1–4) and five ligands (Delta-like (Dll)1, 3, 4, and Jagged 1 and 2) in mice [16, 17]. Because Notch signaling regulates many cellular processes during development, disruption in this signaling pathway is associated with various pathological processes, including inflammatory diseases [18]. Current knowledge in the literature suggests that the Notch pathway is a critical regulator of inflammation [19]. Studies show a relationship between Notch signaling and inflammation-induced PTL [20].

Pregnancy involves inflammatory processes in gestational tissues [21], and a significant proportion of cases of spontaneous PTL is often associated with intrauterine infections within the gestational tissues [22] that can trigger a sequence of events leading to acute maternal vascular malperfusion of the placental bed [23]. In the lipopolysaccharide (LPS)-induced preterm labor model, pregnancy loss within 4 h after LPS administration was associated with structural abnormalities in uteroplacental vascularization, reduced placental blood flow, coagulopathy, placental/fetal hypoxia, and intrauterine fetal death. Disturbances in placental perfusion are responsible for fetal morbidities caused by abnormal maternal inflammation [24]. A necessary inflammatory process that triggers preterm labor is increased prostaglandins (PGs) such as PGE_2 and $PGF_{2\alpha}$ to induce cervical ripening. Prostaglandins are essential for cervical ripening in LPS-mediated PTL but not the term or antiprogestin-driven preterm ripening [25]. The maternal uterine and fetal placental tissues can synthesize PGs that can cross the placenta [26]. While indomethacin (IND) is a PGE_2 inhibitor [27], SP-A is a PGF_{2a} inhibitor [28]. SP-A mutant mice are prone to infection [29], and there are studies in the literature regarding the potential of SP-A to prevent PTL [30]. Studies on Sp-A expression in placental compartments are missing in the literature, and the role of Sp-A in PTL is not clear.

Based on this information in the current literature, this study investigates whether indomethacin delays PTL by regulating the Notch pathway, Tlr receptors, and Sp-A in the placenta in the LPS-induced PTL model.

Materials and methods

Experimental design

Thirty-six virgin female CD-1 mice ($n = 6$ mice in each experimental group) were used. The day when the vaginal plug was seen was considered embryonic day 0.5 (E0.5). Pregnant mice were distributed to pregnant control (PC), surgical abdominal incision applied only (Sham), phosphate-buffered saline (PBS)–administrated, indomethacin (IND)-administrated, lipopolysaccharide (LPS)-administrated, and LPS and IND (LPS + IND)–administrated groups (Fig. 1). Intraperitoneal (i.p.) IND was administered on E14.5 days of pregnancy when the placenta maturation is completed in mice [31]. Moreover, the transition from M1 to M2 macrophages occurs during this period [32]. To initiate preterm labor, 25 µg/100 µl LPS (lipopolysaccharides-*Escherichia coli* O111:B4; L2630-Sigma-Aldrich) was administered intrauterine (IU) to pregnant mice. Two milligrams per kilogram per mouse IND (I7378; Sigma-Aldrich) was applied i.p. for 2 days. The IND injection was done twice with an interval of 24 h. The second daily dose of IND was given to the LPS + IND group 5 h before LPS administration. This dose was chosen since it is less harmful [33]. The mean half-life of IND is estimated to be about 4.5 h [34], and this period has been considered. The total volume for each application was 100 µl in PBS (10010023, 1×, the Ca^{++}, Mg free, pH 7.4, Gibco; Thermo-Fisher). Mice were anesthetized with 0.016 ml/g bodyweights of Avertin dissolved in PBS (2.5% tribromo ethyl alcohol, T48402; Sigma-Aldrich and 2.5%

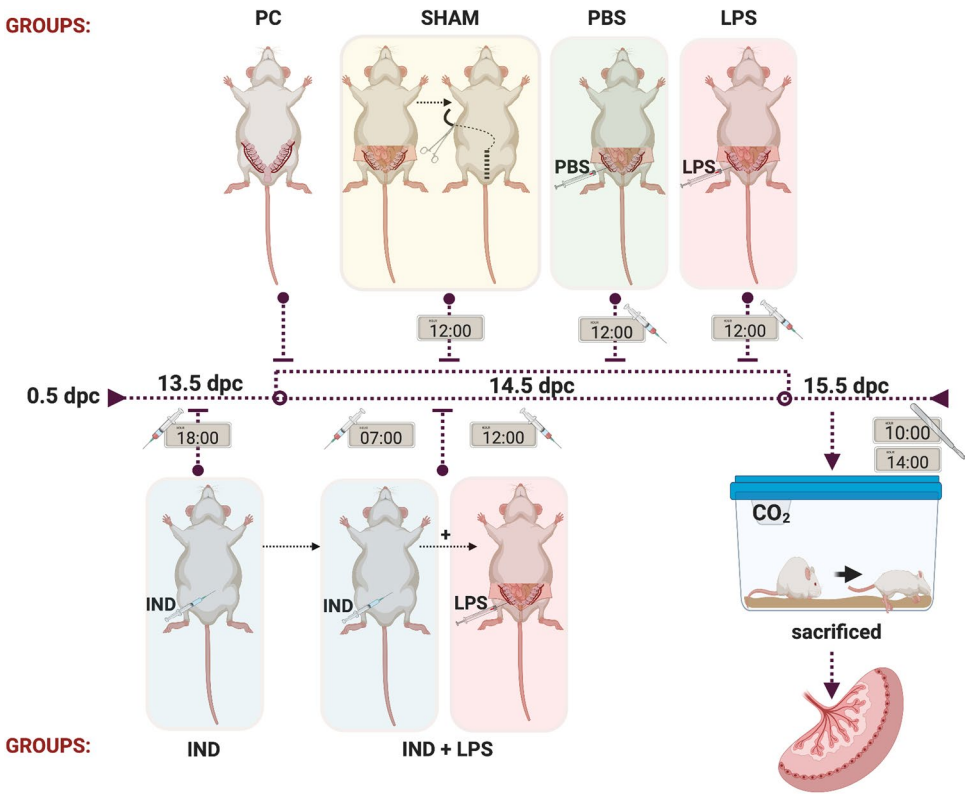

Fig. 1 Experimental design. PC: pregnant control group, Sham: only surgical abdominal incision group, PBS: phosphate-buffered saline group, IND: indomethacin group, LPS: lipopolysaccharide administrated group, LPS + IND: LPS and IND administrated group. dpc day post coitum

2-methyl-2-butanol, 240486; Sigma-Aldrich). Before use, the working solution was filtered through a 0.22-µm sterile filter (SLGV033RS; Merck-Millipore). All IU applications were implemented between two gestational sacs closest to the cervix, and 5.0 Vicryl (Ethicon) was used for surgical suture procedures. The half-life of LPS has been determined as approximately 12 h [35]. Births were observed within 12–24 h after LPS administration. Subsequently, the placentae were removed (E15.5).

Exclusion criteria

For all subjects to have similarities in terms of anesthesia conditions, number of puppies, and weight conditions, the exclusion criteria specified in Table 1 were applied. For this purpose, considering that weight-related differences may indicate nutritional problems or an increase in the fat ratio, subjects with a weight difference of ± 25% (g) more than the average pregnant weight (39.5 g) were excluded from the experiment. Subjects with less than 3 l per uterine horn were excluded because they were outside the overall mean (four and above). In addition, mice that gave birth to less than 80% due to possible problems during LPS administration were not included in the study. According to the criteria explained above, the number of all subjects excluded was three and belonged to the NPC, PC, and LPS groups, respectively. The tissues from excluded subjects were not used in the experiments. No exclusion criteria were applied after tissue harvest.

Immunohistochemical analysis

Placenta tissues were fixed in 10% formalin for 24 h (818708; Merck). Tissues were washed with tap water for 3 h, and then, they were dehydrated in a graded series of ethanol (70, 80, and 90%). Followed by the incubation in 100% ethanol (459844; Sigma-Aldrich) for 3 h, the tissues were then incubated in Xylene (534056; Sigma-Aldrich) for about 5 min. After embedding in paraffin, the tissue blocks were cut into serial sections with a thickness of 5 µm. PBS (P4417-100TAB; Sigma-Aldrich) solution was used for washing the sections. In a microwave, antigen retrieval was performed in citrate buffer (100244; Merck). Three percent of H_2O_2 solution (106009; Merck, 18312; Sigma) was used to remove endogenous peroxidase activity. Ultra V Block (TA-125-UB Thermo Scientific) was performed for 7 min at room temperature. The following primary and secondary antibodies were used: cyclooxygenase 1 (Cox-1) (Abcam, 109025; 1/150), Anti-Sp-A (Abcam, 115791; 1/250), Notch-1 (Abcam, 8925; 1/300), Dll-1 (Abcam, 10554; 1/300), Jagged-2 (Abcam, 109627; 1/200), Tlr-2 (Thermo, PA5-11592; 1/200), Tlr-4 (Abcam, 13556; 1/250), goat anti-rabbit (Vector, BA-1000; 1/500), and rabbit IgG as isotype control (Vector, I-1000). Diaminobenzidine (D4168;

Table 1 Exclusion criteria

No	Group Name	n	Average Weight (gr)	Aplication Type	Surgery	Avertin %2.5 (~540-670 ul) (IP)	Aplication Time LPS (IU)	Aplication Time IND (IP)	Observation Time	Giving Birth	Status of Pups
1	Pregnant Control	6	37		(-)	(-)				0%	Live
2	Sham	6	40,5		(+)	(+)				0%	Live
3	PBS (100 ul)	6	39		(+)	(+)				0%	Live
4	LPS (25 ug/100 ul)	6	38,9		(+)	(+)	11-12 AM		08:00 AM	100%	Dead
5	Indomethacin Control (2 mg/kg)	6	41,8		(-)	(-)		18:00 PM 07:00 AM	08:00 AM 14:00 PM	0%	Live
6	LPS (25 ug/100 ul) + IND (2 mg/kg)	6	39,9		(+)	(+)	11-12 AM		08:00 AM 14:00 PM	66%	Fresh

Reasons for exclusion:
1. Weight : The average weight of the mouse is outside (+/-) 25% in the group's;
2. Number of Pups : If the number of offspring in a uterus is less than 3;
3. Adverse Conditions : Introduction to late anesthesia / excessive anesthetic application;
4. Insufficient LPS stimulation : The number of early birth pups is less than 80% of the total number of offspring;

Sigma) was used as chromogen, and the sections were counterstained with Mayer's hemalum solution (109249, Merck). Sections were examined with an Olympus CX43 Microscope (Japan), and images were captured.

Image analysis and statistical analysis

For immunohistochemical staining intensity analysis, protein expression levels were compared with Image J (1.52 R, National Institutes of Health, USA) software. In detail, while performing Image J analysis, the image type was 8 bits, and the "area" and "area fraction" were marked in the "analyze" and "set measurements" section. To eliminate the non-specific background on the image, the foreground and background were whitened by drawing with the Image J polygonal tool. Then, the stained area was thresholded to analyze and measure the % area values for comparisons. Background subtraction was performed by selecting "process" "subtract background" options, and the accurate separation of the background and the immune-stained area was checked with "rolling ball radius," "1000 px," and "preview" options. The immunohistochemical data presented show the localization for each protein in the placental zones that show specific expression for a marker. We have defined the placental zones by placing dotted lines or solid lines on the figures to distinguish the three different compartments.

Fiji 2021/V.2.3.0/1.53f was used to measure immunohistochemical staining intensity in placental zones. After color deconvolution, a binary image was created. After watershed selection, spots stained by particle analysis were analyzed in the 0.0002-infinity range, and % area values were calculated.

Chi-square test was used to evaluate the differences between LPS and LPS + IND groups in terms of preterm labor delay. For placental mid-sagittal measurements, the groups' differences were determined using ANOVA and the post hoc Sidak test to compare multiple variables for all groups. A randomly selected mid-sagittal placenta photograph was used from six different animals. 344.18 pixels corresponding to 100 µm was used to standardize placental mid-sagittal dimension measurements. For immunohistochemistry evaluations, the groups' differences were determined using repeated measures ANOVA and post hoc Sidak test to compare multiple variables for all groups. Three randomly selected photographs from six different animals in each experimental group were analyzed. Mean ± SD values were presented in the graphics. Differences between groups were statistically significant when the p value was < 0.05. Different letters in the corresponding column indicated significant differences between the groups. GraphPad Prism 8 program was used for statistical analysis.

BioRender program was used in the drawings

Findings

Cox-1 expression in the LZ increased after LPS injection, and IND administration prevented this increase

In the PC, Sham, PBS, and IND groups (Fig. 2a–h); Cox-1 expression was present in the white blood cells in the spiral arteries in the JZ (yellow arrow) while SpT cells (light blue arrows), parietal giant cells (green arrow), trophoblast GCs (dashed red line), and placental villus (dashed blue line) were negative for Cox-1 expression. Cox-1 expression was present in the endothelium of fetal blood vessels (orange arrows) in the labyrinth zone, whereas trophoblast cells did not express Cox-1 (black arrows) (Fig. 2a–h).

In the LPS group, the LZ was necrotic (Fig. 2i–j; below the dashed green line). Cox-1 expression increased significantly in the placenta, especially in the LZ and decidual zone (DZ) (right to the dashed yellow line) (Fig. 2i–j) compared to the PC, Sham, PBS, and IND groups ($p = 0.0004$, $p = 0.0002$, $p = 0.0005$, and $p = 0.0016$, respectively). Its expression was high in the lymphocyte infiltrated regions of the placenta (Fig. 2j; yellow arrows). Expression of Cox-1 was high in the endothelial cells of the fetal blood vessels (orange arrows) and trophoblast GCs (dashed red line). In contrast, maternal blood vessel–associated trophoblasts and SpT cells (light blue arrows) were negative for Cox-1 expression (black arrows) (Fig. 2i–j).

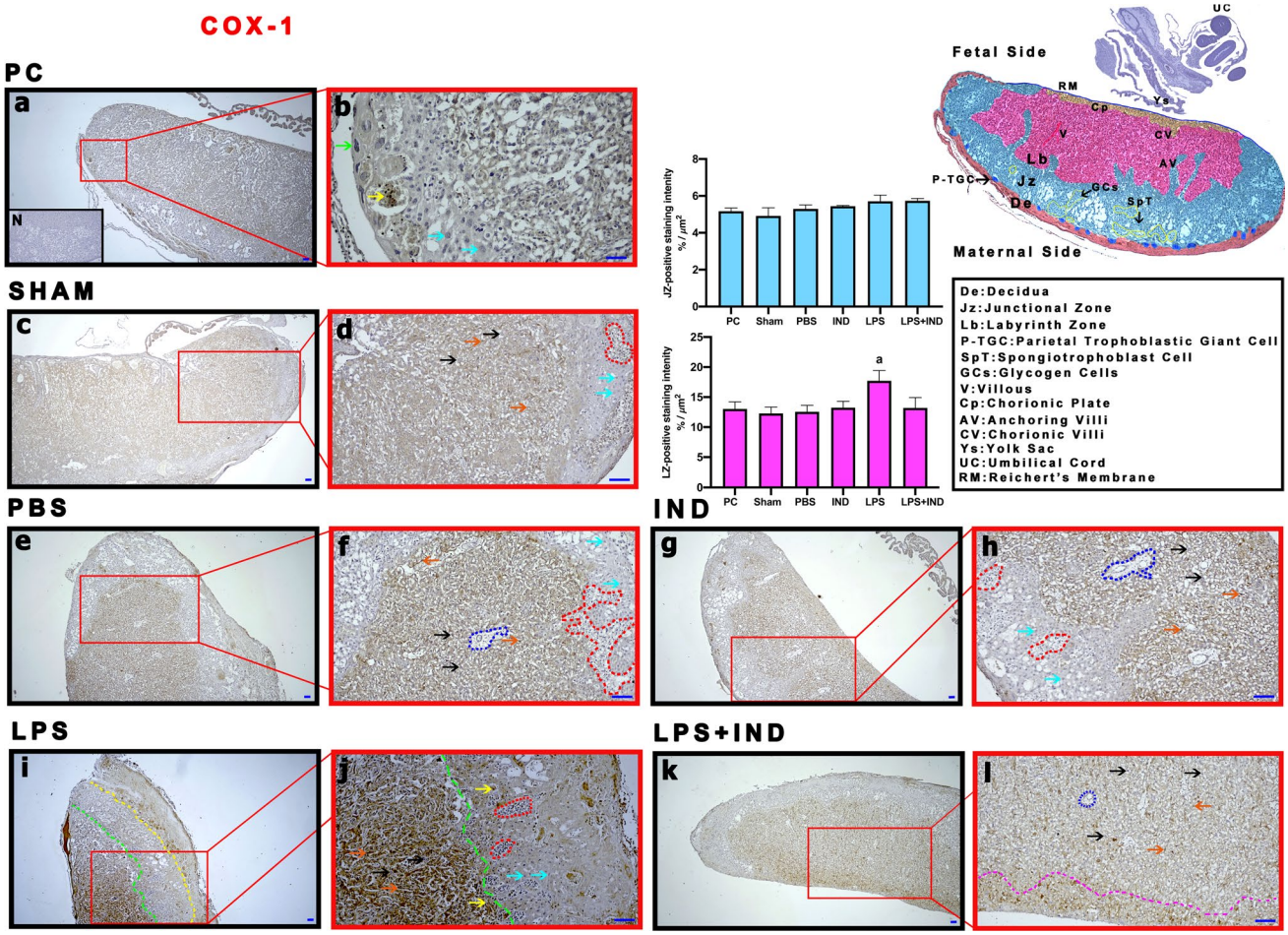

Fig. 2 Cox-1 expression in the LZ increased after LPS injection, and IND administration prevented this increase. Cox-1 expression was present in the endothelium of fetal vessels (orange arrows) in the LZ, whereas sinusoidal trophoblast (black arrows) did not express Cox-1. Cox-1 expression was present in the white blood cells in the spiral arteries in the junctional zone JZ (yellow arrow). In contrast, spongiotrophoblast cells (light blue arrows), parietal giant cells (green arrow), trophoblast glycogen cells (dashed red line), and placental villus (dashed blue line) were negative for Cox-1 expression. The LZ is marked below the green dashed line, the decidual zone is marked above the yellow dashed line, and the chorionic plate is marked below the pink dashed line. Scale bars: 50 μm. [a]The significant differences between the groups were indicated by different letters in the corresponding column

In the LPS + IND group, the boundaries between the placental zones were better distinguishable, and placental morphology was well protected (Fig. 2k). Cox-1 expression increased in the chorionic plate (Fig. 21; below the dashed pink line). Cox-1 expression decreased significantly in the placenta, especially in the LZ compared to the LPS group ($p = 0.013$) (Fig. 2k). Endothelium of fetal blood vessels was positive for Cox-1 expression (orange arrows), while no Cox-1 expression was observed in maternal blood vessel–associated trophoblasts (black arrows) and placental villus (Fig. 2l; dashed blue line).

Sp-A expression in the LZ and JZ decreased after LPS injection, and IND administration prevented this decrease

In the PC, Sham, PBS, and IND groups (Fig. 3a–h), Sp-A expression was present in the sinusoidal trophoblasts (Fig. 3a–h; black arrows) and placental villous vessel endothelium (Fig. 3a–h; dashed blue line) while trophoblast GCs (dashed red line) were negative for Sp-A expression.

In the LPS group, Sp-A expression decreased significantly in the placenta, especially in the LZ compared to the PC, Sham, PBS, and IND groups ($p < 0.0001$ for all comparisons) (right to the dashed green line) (Fig. 3i–j). Its expression was high in the blood cells in the maternal vessels (Fig. 3j; yellow arrows) and in Hoffbauer cells in the JZ of the placenta (Fig. 3i–j; pink arrows), while maternal

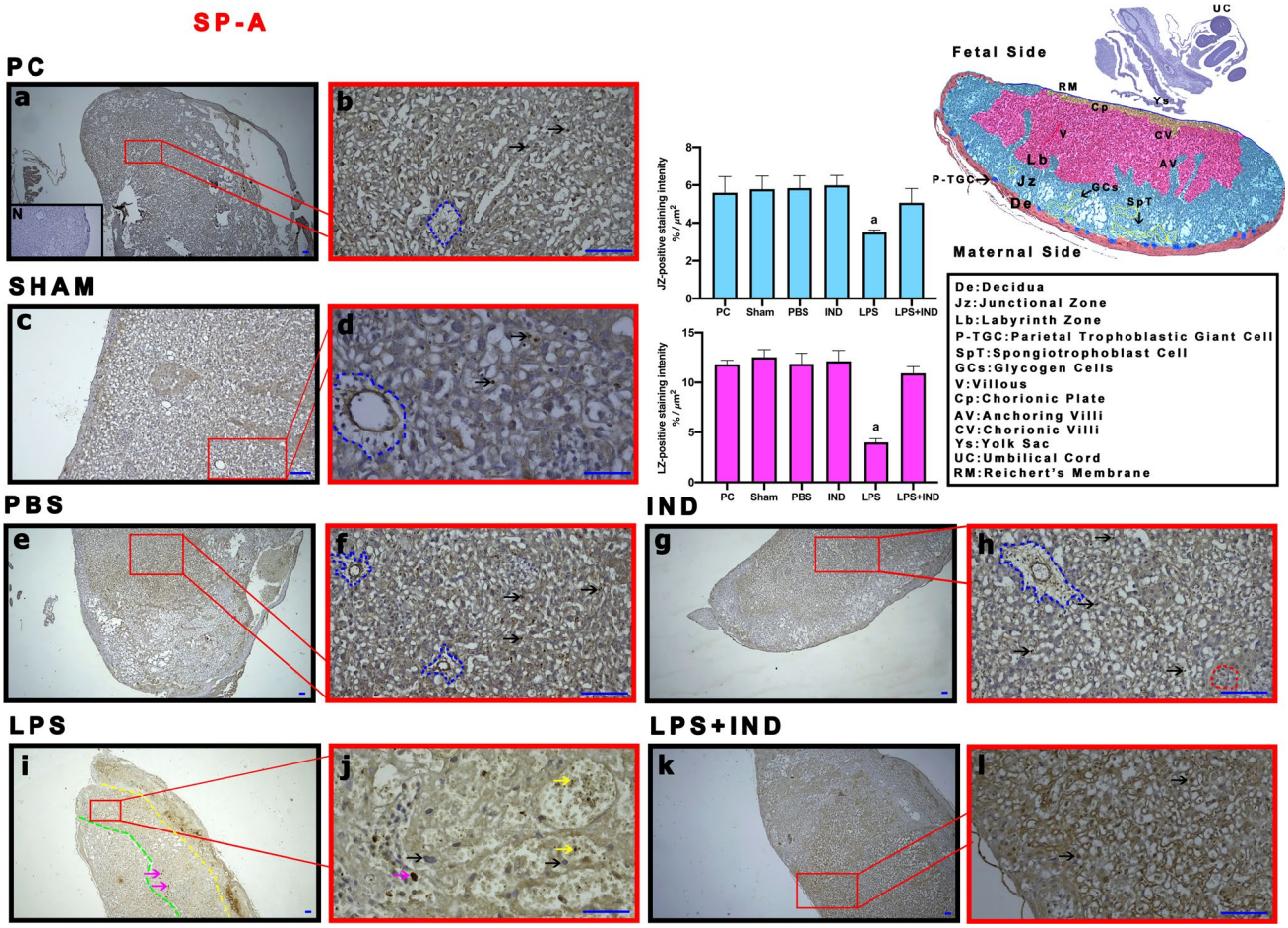

Fig. 3 Sp-A expression in the LZ and JZ decreased after LPS injection, and IND administration prevented this decrease. Sp-A expression was present in the sinusoidal trophoblasts (black arrows) and placental villous vessel endothelium (dashed blue line). In contrast, glycogen cells (dashed red line) were negative for Sp-A expression. Its expression was high in the blood cells (yellow arrows) in the maternal vessels and Hoffbauer cells in the junctional zone of the placenta (pink arrows). Labyrinth zone is marked below the green dashed line, the JZ is marked below the yellow dashed line, and the decidual zone is marked above the yellow dashed line. Scale bars: 50 µm. [a]The significant differences between the groups were indicated by different letters in the corresponding column

blood vessel–associated trophoblasts were negative for Sp-A expression (black arrows) (Fig. 3i–j).

In the LPS + IND group, Sp-A expression increased significantly in the placenta, especially in the LZ trophoblasts and chorionic plate compared to the LPS group ($p < 0.0001$) (Fig. 3k–l; black arrows).

N1 expression in the LZ and JZ increased after LPS injection, and IND administration prevented this increase

In the PC, Sham, PBS, and IND groups (Fig. 4a–h), N1 expression was present in trophoblast GCs in the JZ (Fig. 4a–h; dashed red line).

In the LPS group, N1 expression increased significantly in the placenta, especially in the LZ trophoblast GCs, compared to the PC, Sham, PBS, and IND groups ($p <$ 0.0001 for all comparisons) (Fig. 4i–j dashed red line). N1 expression was also present in the LZ (Fig. 4i; below the dashed green line) and the chorionic plate (Fig. 4i; below the dashed pink line).

In the LPS + IND group, N1 expression was present in the JZ in the trophoblast GCs similar to the PC, Sham, PBS, and IND groups, and its expression level significantly in the placenta compared to the LPS group ($p < 0.0001$) (Fig. 4l; dashed red line).

Dll-1 expression in the LZ and JZ increased after LPS injection, and IND administration prevented this increase

In the PC, Sham, PBS, and IND groups (Fig. 5a–h), Dll-1 expression was present in the LZ in trophoblast cells (Fig. 5d, h; black arrows) and in JZ in SpT (Fig. 5b, f; light blue arrows) and in few trophoblast GCs in the JZ

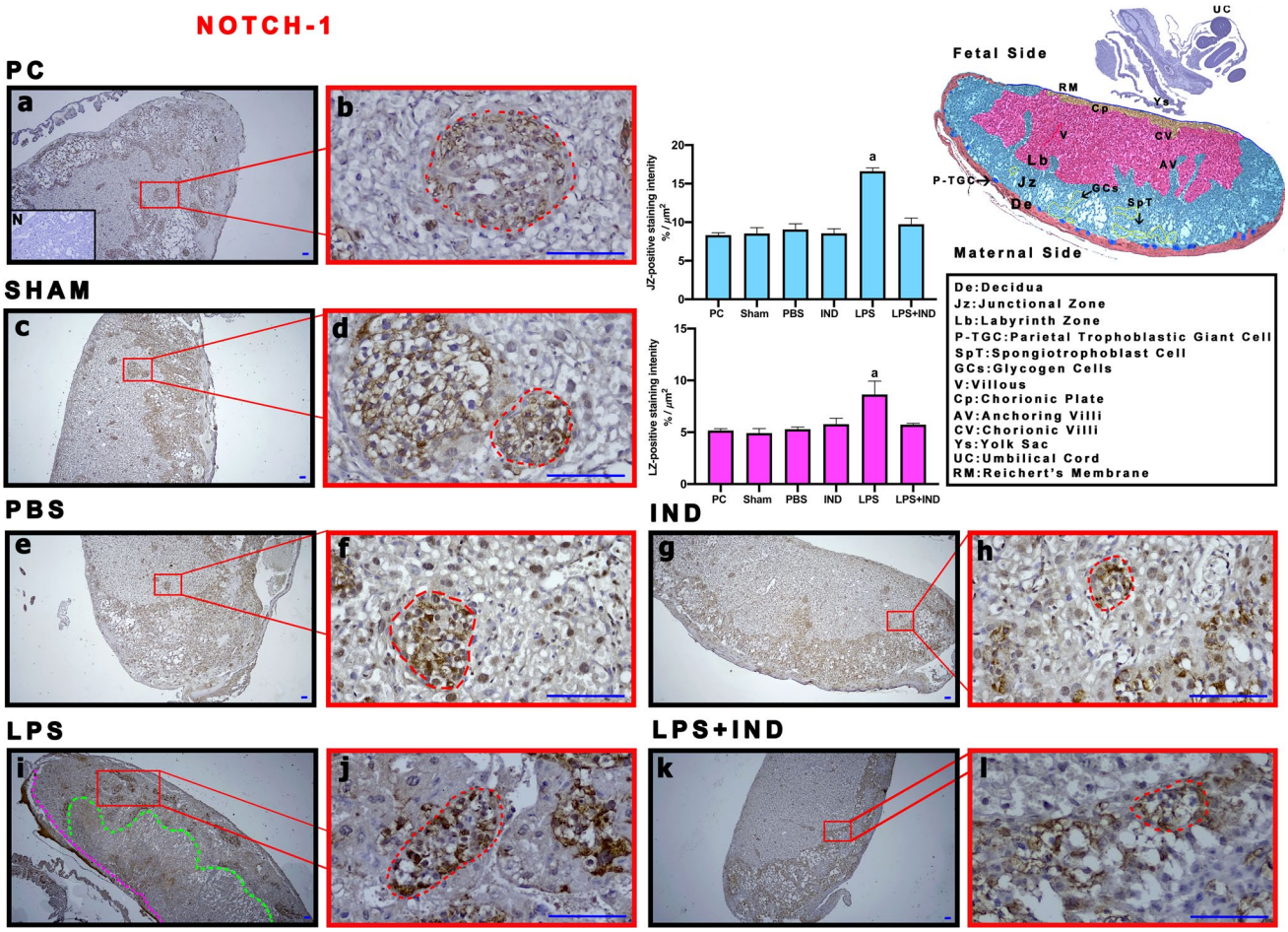

Fig. 4 N1 expression in the LZ and JZ increased after LPS injection, and IND administration prevented this increase. N1 expression was present in glycogen cells (dashed red line) in the JZ (above the green dashed line) of the placenta. N1 expression was also present in the LZ (below the green dashed line) and the chorionic plate (dashed red line). Scale bars: 50 μm. [a]The significant differences between the groups were indicated by different letters in the corresponding column

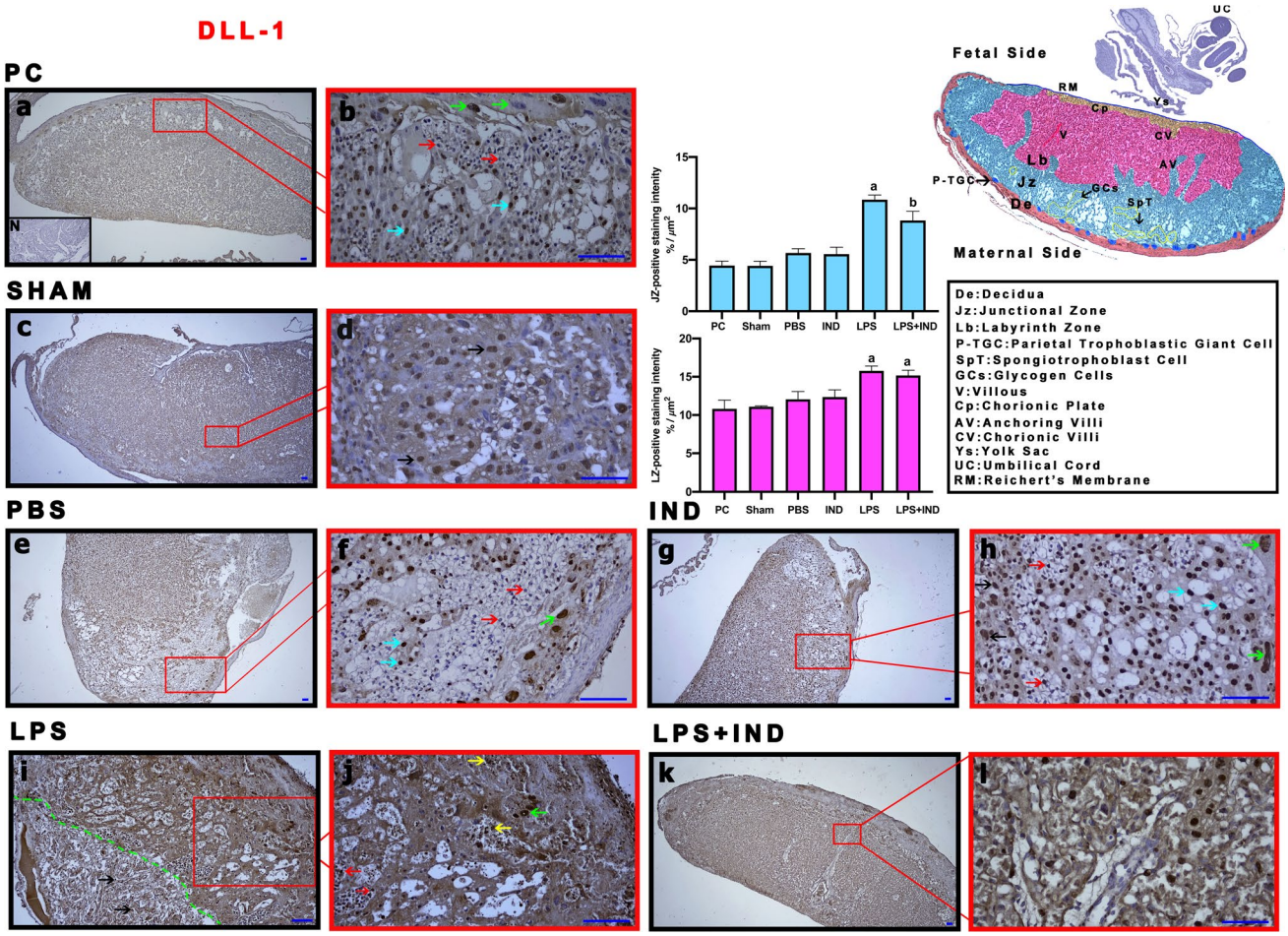

Fig. 5 Dll-1 expression in the LZ and JZ increased after LPS injection, and IND administration prevented this increase. Dll-1 expression was present in the LZ in trophoblast cells (black arrows) and JZ in spongiotrophoblast (light blue arrows), and a few glycogen cells (red arrows). Some parietal giant cells were positive for Dll-1 (green arrows). Dll-1 expression increased in vasodilated and lymphocyte infiltrated (yellow arrow) maternal vessels. The LZ is marked below the green dashed line, and the JZ is marked above the green dashed line. Scale bars: 50 µm. [a,b]The significant differences between the groups were indicated by different letters in the corresponding column

(Fig. 5a–h; red arrows). Some (P)-TGCs were positive for Dll-1 (Fig. 5b, f, h; green arrows).

In the LPS group, Dll-1 expression increased in the placenta, especially in the trophoblast GCs in the JZ (Fig. 5i–j) and in sinusoidal trophoblasts in the LZ (Fig. 5i; black arrows), and in (P)-TGCs in decidua (Fig. 5j; green arrow), and (Fig. 5j; red arrows) compared to the PC, Sham, PBS, and IND groups ($p < 0.0001$ for all comparisons). Dll-1 expression also increased in vasodilated and lymphocyte infiltrated maternal vessels (Fig. 5j; yellow arrows).

In the LPS + IND group, Dll-1expression decreased significantly in the placenta, especially in the JZ, compared to the LPS group ($p < 0.0001$) (Fig. 5k, l).

Jag-2 expression in the JZ increased after LPS injection, and IND administration diminished this increase

In the PC, Sham, PBS, and IND groups (Fig. 6a–h), Jag-2 expression was present in trophoblasts (Fig. 6d; black arrows), SpT (Fig. 6b, h; light blue arrows), (P)-GTCs (Fig. 6b, f, h; green arrows), and trophoblast GCs (Fig. 6f, h; red arrows). Moreover, high Jag-2 expression was present in fetal vessels (Fig. 6d; dashed orange line) while endothelium was negative for Jag-2 expression (Fig. 6d; orange arrows).

In the LPS group, Jag-2 expression increased significantly in the JZ compartment compared to the PC, Sham, PBS, and IND groups ($p < 0.0001$ for all comparisons) (Fig. 6i–j). The

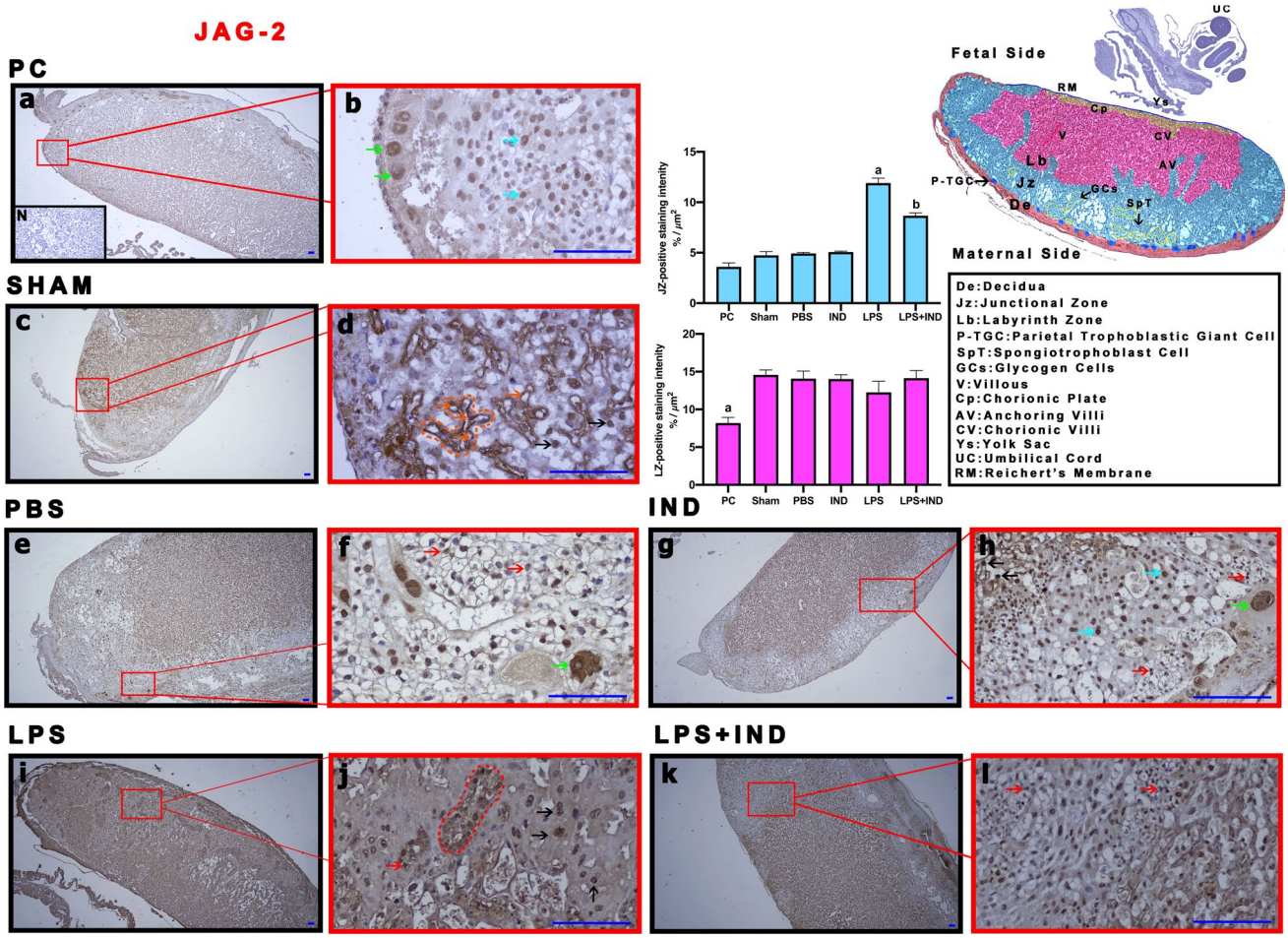

Fig. 6 Jag-2 expression in the JZ increased after LPS injection, and IND administration diminished this increase. Jag-2 expression was present in trophoblasts (black arrows), spongiotrophoblast (light blue arrows), parietal giant cells (green arrow), and glycogen cells (red dashed line and arrow). Moreover, high Jag-2 expression was present in fetal vessels (dashed orange line) while vessel endothelium was negative for Jag-2 expression (orange arrows). Scale bars: 50 μm. [a,b,c]The significant differences between the groups were indicated by different letters in the corresponding column.

Jag-2 expression also increased in trophoblast GCs (Fig. 6j; red arrow and dashed red line).

In the LPS + IND group, Jag-2 expression decreased significantly in the placenta, especially in the JZ, compared to the LPS group ($p < 0.0001$) (Fig. 6k, l). The Jag-2 expression also decreased in trophoblast GCs (Fig. 6l; red arrows).

Tlr-2 expression in the LZ and JZ increased after LPS injection, and IND administration prevented this increase

In the PC, Sham, PBS, and IND groups (Fig. 7a–h), Tlr-2 expression was present in LZ Fig. 7a, c, e, g) and in some trophoblast GCs (Fig. 7b, d red arrows) in the junctional zone. Its expression was also present in some leukocytes in maternal blood vessels (Fig. 7f; yellow arrows).

In the LPS group, Tlr-2 expression increased significantly in the placenta, especially in the LZ, in trophoblast GCs (Fig. 7j; dashed red line), and in leukocytes in maternal blood vessels (Fig. 7j; yellow arrows) compared to the PC, Sham, PBS, and IND groups ($p < 0.0001$ for all comparisons) (Fig. 7i; left to the dashed green line).

In the LPS + IND group, Tlr-2 expression decreased significantly in the placenta, especially in the LZ, compared to the LPS group ($p < 0.0001$) (Fig. 7k, l).

Tlr-4 expression in the JZ increased after LPS injection, and IND administration diminished this increase

In the PC, Sham, PBS, and IND groups (Fig. 8a–h), Tlr-4 expression was present in SpT cells in LZ (Fig. 8b, d, f, h; light

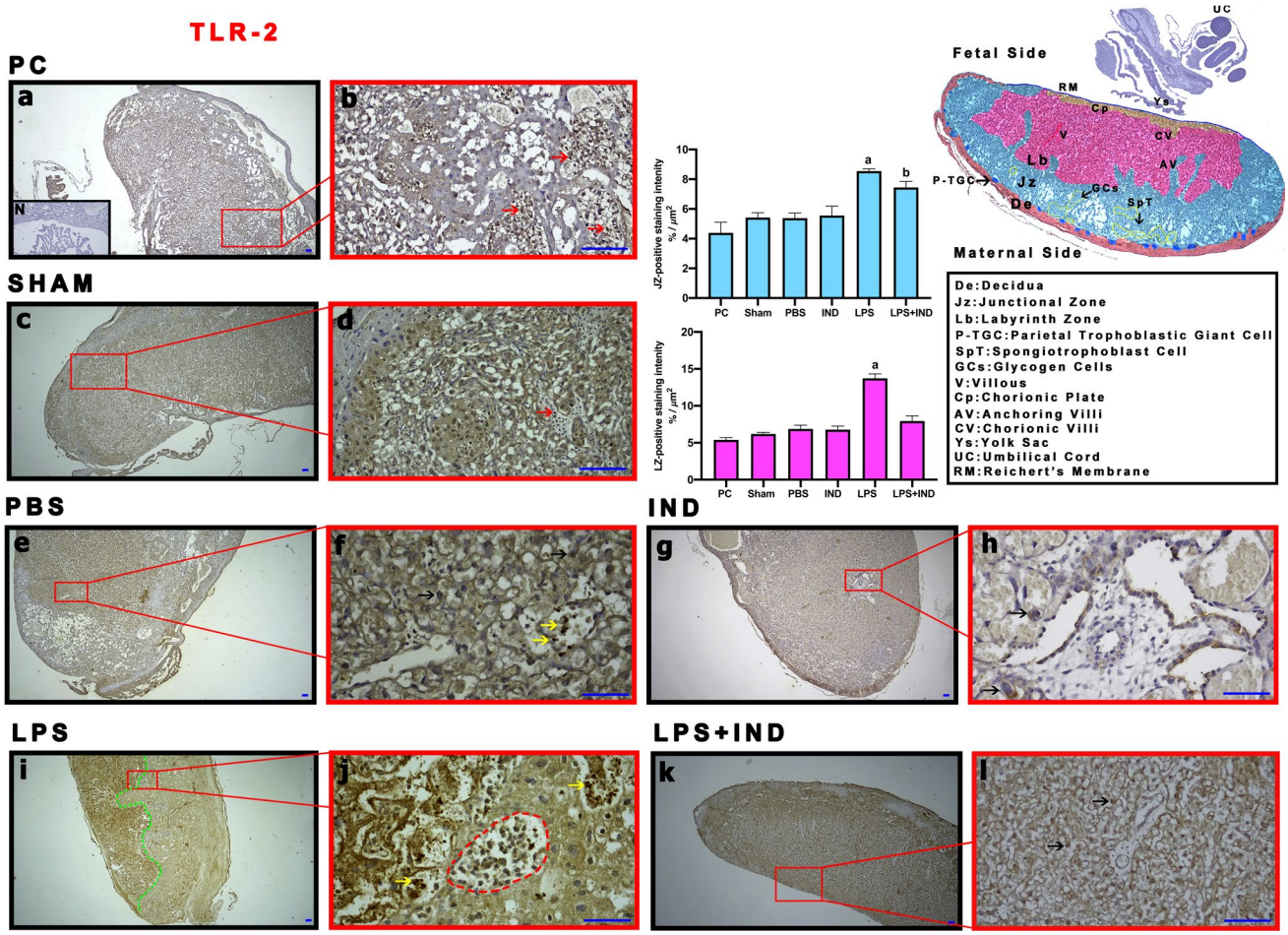

Fig. 7 Tlr-2 expression in the LZ and JZ increased after LPS injection, and IND administration prevented this increase. Tlr-2 expression was present in sinusoidal trophoblasts (black arrow) and some glycogen cells (red dashed line and arrow). Tlr-2 expression increased in leukocytes (yellow arrow) in maternal blood vessels. The LZ is marked below the green dashed line, and the JZ is marked above the green dashed line. Scale bars: 50 μm. [a,b]The significant differences between the groups were indicated by different letters in the corresponding column

blue arrows) and trophoblast GCs (Fig. 8b, d, f; red arrows) in the junctional zone.

In the LPS group, Tlr-4 expression increased significantly in the placenta, especially in the JZ trophoblast GCs (Fig. 8j; dashed red line), SpT (Fig. 8j; light blue arrows), Hoffbauer cells (Fig. 8j; pink arrows) and in (P)-TGCs (Fig. 8j; green arrow) compared to the PC, Sham, PBS, and IND groups ($p < 0.0001$ for all comparisons) (Fig. 8i; left to the dashed green line).

In the LPS + IND group, Tlr-4 expression decreased significantly in the placenta, especially in the JZ and in trophoblast GCs (Fig. 8l; dashed red line), compared to the LPS group ($p < 0.0001$) (Fig. 8k, l).

Indomethacin delays preterm delivery induced by LPS and preserves placental morphology

All pups in the PC, Sham, and PBS groups were alive at birth, while all were stillborn in the LPS and LPS + IND groups. On the other hand, offspring and placentae in the LPS + IND groups had a fresh and non-hemorrhagic appearance compared to the LPS group on macroscopic observation. In the LPS-induced PTL model, the birth rate in LPS-treated mice was 100%, and all pups were stillborn. The PTL rate in the LPS + IND group, observed simultaneously as the LPS-only group, was 33%. When the mothers in the LPS group completed preterm labor, bleeding was observed

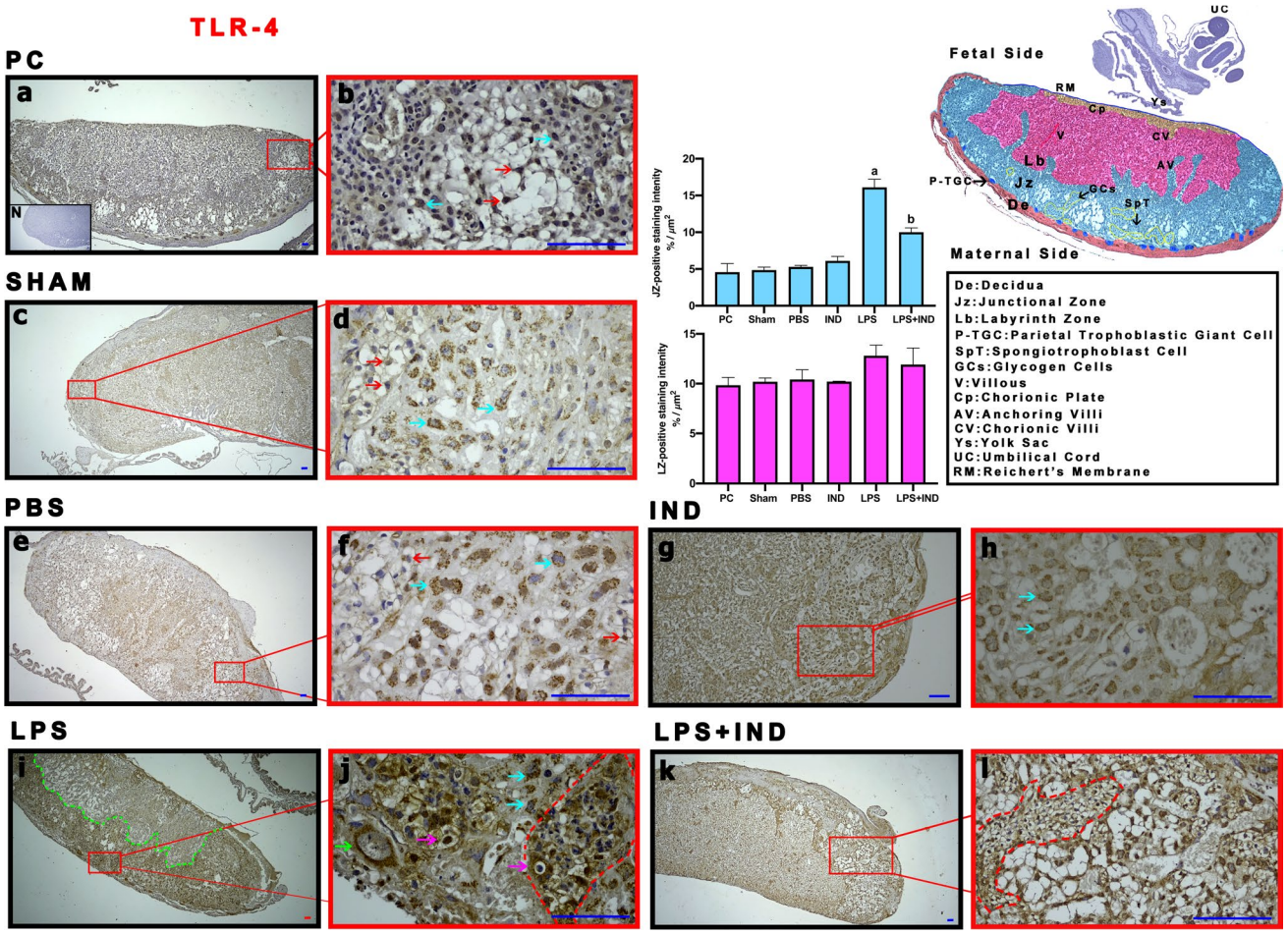

Fig. 8 Tlr-4 expression in the JZ increased after LPS injection, and IND administration diminished this increase. Tlr-4 expression was present in spongiotrophoblast cells (light blue arrows) in LZ (above the green dashed line) and glycogen cells (red dashed line and red arrows) in the JZ. In the LPS group, Tlr-4 expression increased in the JZ (above the green dashed line) and in Hoffbauer cells (pink arrows), and in parietal giant cells (green arrow). Scale bars: 50 μm. [a,b]The significant differences between the groups were indicated by different letters in the corresponding column.

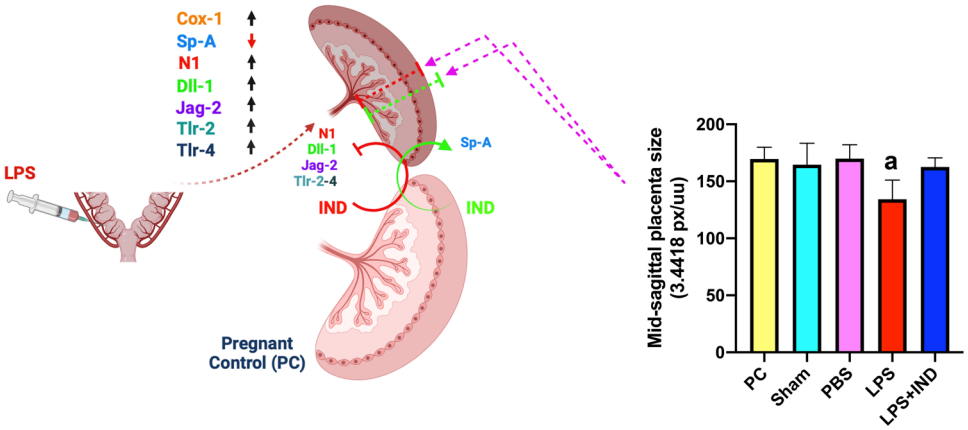

Fig. 9 Possible effect of indomethacin on Notch signaling pathway, Tlr receptors, and Sp-A expression and placental size in LPS-induced PTL model in mice. PC: pregnant control group, Sham: only surgical abdominal incision group, PBS: phosphate-buffered saline group, IND: indomethacin group, LPS: lipopolysaccharide administrated group, LPS + IND: LPS and IND administrated group

for 5–7 h in the mothers who had not yet given birth (66%) in the LPS + IND group. At the end of this period, 66% of mothers in the LPS + IND group gave birth prematurely. Therefore, it was concluded that IND delayed preterm labor due to LPS induction by at least 5 h. In LPS-treated mice, placental mid-sagittal size measurements were significantly

reduced, whereas placental mid-sagittal measures in the LPS + IND group were similar to the PC group (Fig. 9).

Discussion

In the present study, we evaluated the expression of the Notch signaling pathway, Tlr2, Tlr4 receptors, and Sp-A in the placenta in the LPS-induced PTL model after IND administration. We found that PG inhibition may delay preterm labor by altering the expression of Notch pathway, Tlr receptors, and Sp-A in different placental zones. On the other hand, while placental morphology was impaired after LPS induction, administration of IND with LPS induction prevented this deterioration.

Abnormal inflammation that develops 24 h after LPS administration during pregnancy is associated with spontaneous pregnancy loss and intrauterine fetal growth restriction (FGR), resulting in severe bleeding and fibrin deposition in the uterus [24]. The maternal LPS administration has been associated with fetal hypoxia, increased fetal cortisol levels, and PG secretion [36]. In addition, fetal placental membranes provide a physical barrier against microbial invasion due to LPS administration and protect the intrauterine environment [37] and are therefore a critical determinant of fetal death [24]. A study observed that the placental volume in the LPS-induced mouse model was 13% lower than in control placentae [23]. We also found that placental morphology was impaired after LPS induction; however, IND administration prevented this deterioration.

Indomethacin, a non-specific Cox inhibitor, blocks PGE_2 production and is a nonsteroidal anti-inflammatory drug with antipyretic and analgesic effects [38]. Cox-1 is constitutively expressed in most tissues and regulates housekeeping functions such as platelet aggregation and homeostasis [39]. COX-1 action is required for the appropriate timing of term labor in mice, and Cox-1 mRNA increases markedly during late gestation in mouse uterus. Basal PG production in the mouse uterus is predominantly attributable to Cox-1, and LPS causes a twofold rise in uterine PG. Specific COX-1 inhibition may reduce PTL by inhibiting basal PG synthesis [40, 41]. Although studies in the literature emphasize the importance of COX-2, especially in PTL associated with inflammation, the mechanisms related to the balance of expression between COX-1 and COX2 in placental pathologies are still unclear. In the present study, we found that Cox-1 expression in the LZ zone increased after LPS injection, and IND administration prevented this increase. Cox-1 expression in the LZ in the LPS group may increase due to inflammatory damage and thus may be trying to balance this pathological condition. Furthermore, IND administration remarkably diminished the increase of Cox-1 expression in the vascular LZ. The underlying mechanism needs to be investigated further.

Notch receptors and ligands are also involved in the formation of vascular structures and embryo survival [10]. Our study revealed that N1 expression in the LZ and JZ zones increased after LPS injection, and IND administration prevented this increase. Both Dll-1 and Jag-2 expressions in JZ increased after LPS injection compared to the control group, and IND administration prevented their increase. Moreover, expression of Dll-1 increased in LZ after LPS injection, and IND administration prevented this increase. Delta and Jagged have opposing roles in endothelial sprouting. Thus, altering the balance between these ligands can positively or negatively modulate angiogenesis. In angiogenesis, two types of cell transformation occur [42, 43]. The cells that express high delta levels transform into tip cells, while cells that express low delta levels transform into stalk cells. On the other hand, overexpression of Jagged leads to a hybrid tip/stalk phenotype, and the irregular and poorly perfused vessels formed in this phenotype can provide rapid oxygen supply [44]. The increased expression of both Dll-1 and Jag-2 in the placental zones after LPS-induction may be due to the need for immediate oxygen support compatible with the hybrid type/stalk phenotype. When IND is applied together with LPS, the decrease in Dll-1 and Jag-2 expressions can be attributed to the prevention of this type of damage in the placenta and needs further mechanistic investigation.

Notch expression was particularly evident in the trophoblast GCs in the JZ. These cells are the endocrine component of the mouse placenta that store and release glycogen, and increased glycogen storage in trophoblast GCs has been associated with FGR [45]. In our study, increased N1 expression in trophoblast GCs may be associated with increased fetal energy demand after LPS induction which is crucial for fetal survival. Moreover, Notch signaling has a significant role in endocrine development, for example, enhanced Notch signaling in pancreatic progenitor cells impairs the differentiation of these cells into various pancreatic cell lines [46]. In our study, increased N1 expression in trophoblast GCs after LPS induction may impair the differentiation of GCs and may also suggest the presence of decreased fetal energy supply. Prevention of increased N1 expression in JZ after LPS induction by IND administration may be associated with PTL, and mechanistic studies investigating this issue are needed.

Amniochorion membranes can express TLR-1, TLR-2, TLR-4, and TLR-6, and increased TLR-1 and TLR-2 have been associated with chorioamnionitis in PTL [47]. Instead, it has been reported that the expression of Tlr-2 and Tlr-4 in chorioamnionitis increases independently of the amniochorionic membrane [48]. Moreover, a recent study has identified increased expression of Tlr-2 and Tlr-4

in the placenta after LPS induction [49]. We found that Tlr-2 expression increased in the LZ, and Tlr-4 expression increased in the JZ after LPS injection. Thus, in our study, placental zone expression of Tlr-2 and Tlr-4 has been well-defined. Expression of Tlrs in different regions of the placenta may be associated with regulating inflammatory response and maintaining homeostasis. While IND application decreased Tlr-2 and Tlr-4 expression, Tlr-2 suppression was more dramatic than Tlr-4. Altogether, these results suggest that the restorative effect of IND may be mediated primarily by Tlr-2. Since it has been recommended that suppression of Tlr-2 and Tlr-4 expression may effectively prevent PTL [49], based on our findings, it can be also suggested that PG inhibition may prevent PTL through Tlr-2. The underlying immunological mechanism needs to be investigated further. Previous reports suggested that Sp-A upregulates Tlr-2 expression, thereby preventing inflammation-induced PTL via Tlr-2 [30, 50]. It has also been shown that SP-A inhibits LPS-induced cytokine upregulation in vivo [51] and may have a role in the regulation of inflammation [52]. Our results showed that SP-A expression decreased in the LZ of the placenta after LPS administration which may be associated with the occurrence of an inflammatory environment; thus, its decreased expression may cause an upregulated inflammatory state in the maternal-fetal exchange zone. IND treatment prevented this decrease, thus may be associated with delayed PTL via regulating the inflammatory state of LZ that needs further evaluation.

Indeed, our results are essentially based on immunohistochemistry analysis which may be considered as a limitation of the present study. Although it is helpful to determine protein levels by more quantitative methods such as Western blot, we first planned to reveal whether the expressions of the Notch signaling pathway, Tlr-2, and Tlr-4 receptors are altered in different compartments of the placenta after LPS induction. Immunohistochemistry analysis revealed placental zone differences in these proteins' expression and also showed significant variation in expression at the cellular level. The presence of different localizations of Notch signaling pathway proteins and Tlr receptors in both the control and experimental groups highlighted their possible different roles in different placental compartments and well-supported the discussion of our findings.

In conclusion, inhibition of PG may delay LPS-induced preterm labor and help preserve placental morphology by altering Notch pathway, Tlr and SP-A protein expressions. Our findings highlight key evidence for altered expression of these pathways after PG inhibition in the PTL model and shed light on investigating the molecular mechanisms underlying PTL pathogenesis to improve placental function and delay PTL.

Author contribution SA designed the study, performed the experimental procedures, analyzed the data, and drafted the manuscript. NK, BD, and LK assisted SA during experiments, surgical procedures, and data generation. IU acted as a consultant during the execution of the project. CCO contributed to the data analysis and drafting of the article.

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Data availability All data will be provided by the corresponding author upon request.

Declarations

Ethics approval The Animal Research Ethical Committee approved the experimental protocol of Akdeniz University by protocol number 1201/2020.10.008. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate All authors have read and accepted the content of the article; all authors have their consent to participate in the study.

Consent for publication All authors have consent for the publication of this article.

Conflict of interest The authors declare no competing interests.

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