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Research Article

Interleukin 22 prevents lipopolysaccharide-induced preterm labor in mice†

Svetlana Dambaeva[1,*], Sylvia Schneiderman[1], Mukesh K Jaiswal[1], Varkha Agrawal[2], Gajendra K Katara[1], Alice Gilman-Sachs[1], Emmet Hirsch[2,3] and Kenneth D Beaman[1]

[1]Department of Microbiology and Immunology, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois, USA; [2]Department of Obstetrics and Gynecology, NorthShore University HealthSystem, Evanston, Illinois, USA and [3]Department of Obstetrics and Gynecology, Pritzker School of Medicine, University of Chicago, Chicago, Illinois, USA

*__Correspondence:__ Clinical Immunology Laboratory, Department of Microbiology and Immunology, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, IL 60064, USA. Tel: +(847) 578-8843; E-mail: svetlana.dambaeva@rosalindfranklin.edu

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Abstract

Preterm birth is widespread and causes 35% of all neonatal deaths. Infants who survive face potential long-term complications. A major contributing factor of preterm birth is infection. We investigated the role of interleukin 22 (IL22) as a potential clinically relevant cytokine during gestational infection. IL22 is an effector molecule secreted by immune cells. While the expression of IL22 was reported in normal nonpregnant endometrium and early pregnancy decidua, little is known about uterine IL22 expression during mid or late gestational stages of pregnancy. Since IL22 has been shown to be an essential mediator in epithelial regeneration and wound repair, we investigated the potential role of IL22 during defense against an inflammatory response at the maternal–fetal interface. We used a well-established model to study infection and infection-associated inflammation during preterm birth in the mouse. We have shown that IL22 is upregulated to respond to an intrauterine lipopolysaccharide administration and plays an important role in controlling the risk of inflammation-induced preterm birth. This paper proposes IL22 as a treatment method to combat infection and prevent preterm birth in susceptible patients.

Summary Sentence

IL22 is upregulated in uterine tissue in response to bacterial endotoxin and contributes to defense against inflammatory reaction at the maternal-fetal interface

Key words: endometrium, interleukin 22, IL22, pregnancy, preterm birth, uterus.

Introduction

It is estimated that 11% of all live births worldwide are preterm (<37 weeks of gestation) [1]. The preterm birth burden is extensive. It is a single direct cause in 35% of neonatal death cases, while surviving infants often suffer from long-term complications including various physical effects (visual, hearing impairment, chronic lung disease, cardiovascular disease), neurodevelopmental and behavioral deficiencies [2, 3]. The underlying causes of preterm birth vary, but it

is estimated that up to 40% of all preterm births are associated with infections [4]. Bacterial vaginosis and urinary tract infections are common sources of pathogens contributing to intrauterine (IU) infection via an ascending route [5]. Hematogenous spread of pathogens to the maternal–fetal interface and to the placenta is implicated in preterm birth linked to periodontal disease and listeria infection [6]. Young or advanced maternal age, compromised socioeconomic status, high blood pressure during pregnancy, stress, tobacco, alcohol or substance abuse are among risk factors associated with preterm birth [3, 7]. The mechanisms of how these risk factors are linked to preterm birth are not fully understood, although inflammation is thought to be implicated in preterm birth associated with obesity and stress [8].

Animal studies support the role of infection and infection-associated inflammation in the pathogenesis of preterm birth [9–11]. It has been demonstrated that both IU and systemic administration of lipopolysaccharide (LPS) causes preterm birth or IU fetal death in mice [12, 13]. Recognition of LPS via pattern-recognition receptor of the toll-like receptor family, TLR4, and CD14, which are expressed by myeloid cells residing in the uterus (macrophages, monocytes, neutrophils, or dendritic cells), induces increased production of proinflammatory cytokines (e.g., tumor necrosis factor [TNF], interleukin 1 beta [IL1B], IL6) [14]. These cytokines can activate signal pathways leading to upregulation of cyclooxygenase-2 (COX-2) and the increasing release of prostaglandin E2 by myometrial cells leading to uterine contractions [15]. The use of anti-TNF or anti-IL6 decreased fetal loss in an LPS-induced murine model of preterm birth [16, 17]. The abundant expression of interleukin 10 (IL10) in uterine tissues implies a strong regulation of the inflammation [18]. Studies with IL10 null mice proved that uncontrolled production of proinflammatory cytokines results in pregnancy loss [13]. Uterine NK (uNK) cells were shown to play a significant role in mediating these adverse effects in IL10-null mice, while TNF neutralization, NK cell depletion or IL10 supplementation could rescue the pregnancy loss in IL10-/- mice [19, 20].

Intrauterine LPS challenge in mice was reported to be associated with neutrophil infiltration at the maternal–fetal interface; however, at the same time it was shown that the absence of neutrophils did not delay preterm birth [21]. Conversely, others have reported relatively little myometrial and decidual leukocyte infiltration upon IU LPS treatment [22]. A decrease in inflammation-induced preterm birth was shown in mice with depleted invariant NKT cells [23]. A resistance to LPS-induced abortion was observed in interleukin 15 (IL15)-deficient mice [24]. IL15 is a cytokine that is essential for development and activation of NK cells, suggesting that NK cells populating the uterus are involved in the mechanism of preterm birth.

uNK cells are a type of innate lymphoid cell (ILC) residing within myometrial and endometrial stroma and play an important role during early and mid-pregnancy. Studies with transgenic mice, including alymphoid mice and Killer cell lectin-like receptor, subfamily A (*Klra;* previously known as Ly49) knockdown mice, demonstrated that uNK cells are involved in the processes of endometrium decidualization and uterine lumen closure [25, 26]. Interferon gamma (IFNG) production by uNK cells is essential in triggering spiral artery remodeling, a fundamental process that ensures blood supply for the developing embryo [27]. The population of uNK cells is both dynamic and heterogeneous. They are present in the virgin and the preimplantation uterus and their numbers increase substantially at midgestation, when they are abundantly located in decidua interfacing fetal-originated trophoblast cells [28]. The majority of mouse uNK cells can be recognized based on reactivity to lectin *Dolichos Biflorus* agglutinin (DBA) [29]. DBA-reactive uNK cells appear to be unique to the uterus and are functionally biased towards angiogenesis. They have low levels of *Ifng* transcripts, but higher expression of angiogenic factors and *Il22* [30].

IL22 is a unique cytokine secreted by immune cells including Th17 cells, which are implicated in broad array of inflammatory responses, and by ILC, particularly ILC3, that play important roles in host defense at mucosal surfaces and tissue repair [31]. ILC3 are detectable at low frequency in the uterus of virgin and pregnant mice and were shown to constitutively produce IL22 and IL17 [32]. Similarly, ILC3 were identified in human endometrium and decidua (endometrium during pregnancy) [32, 33]. The role of uterine ILC3 as well as other ILCs in reproduction is not elucidated yet, although it is thought that they play a role in innate defense and tissue homeostasis. Interestingly, uNK cells in human endometrium were also reported to express IL22 [34, 35]. IL22 acts on epithelial and parenchymal cells of nonhematopoietic origin at various barrier surfaces and organs such as skin, intestine, lung, liver, and thymus. It promotes proliferation and survival of epithelial cells, as well as induces secretion of antimicrobial peptides [36]. Trophoblast cells are epithelial cells of fetal origin. They were shown to respond to IL22 by increased proliferation and survival [35]. More data are accumulating on the importance of IL22 in epithelial regeneration and wound repair [37, 38]. Upregulation of IL22 in uterine tissues was reported to be in response to intragastric infection of pregnant mice with *Listeria monocytogenes* [39]. However, it was not clear if IL22 upregulation could be related to the pathogenesis of pregnancy loss or it plays a different role.

In the current study, we hypothesized that IL22 could contribute to defense against inflammatory responses at the maternal–fetal interface in response to IU infection. We used a well-established LPS-induced preterm labor model [9, 12, 40] in the mouse to test this hypothesis. We found that IL22 is upregulated in uterine tissue in response to bacterial endotoxin and prevents apoptosis of placental cells. Importantly, supplementation with recombinant IL22 (rIL22) significantly improved the pregnancy outcome in mice that are challenged with IU LPS treatment.

Materials and methods

Mice

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Rosalind Franklin University and by the NorthShore University HealthSystem Animal Care and Use Committee and conform to the Guide for Care and Use of Laboratory Animals (1996, National Academy of Sciences). Mice used in the present studies were C57BL/6J (B6, The Jackson Laboratory Cat# 000664); 129S5-*Il22*tm1.1Lex/Mmucd (*Il22*KO, MMUCD Cat# 036745-UCD), and the CD1 strain (Harlan laboratories, Madison, WI). Female mice in estrus were selected by the gross appearance of the vaginal epithelium [41] and were impregnated naturally. Mating was confirmed by the presence of a vaginal plug and the day of plug formation was counted as day 0.5 of pregnancy. Males were left with females for a maximum of 2 days to ensure accuracy of gestation date.

Animal treatment and tissue harvest

C57BL/6J and *Il22*KO mice

Intrauterine injection of LPS (extracted from *Salmonella enterica*, L2262, Sigma, St. Louis, MO, with doses of 25, 10, 1, or 0.1

μg/animal) was performed into the right uterine horn on day 14.5 of a 19–20-day gestation period, as previously described [9, 40, 42]. In the B6 mouse, the 25 μg intervention reliably induced preterm delivery within 24–48 h, while 10 or 1 μg doses were generally tolerated. The *Il22*KO mouse was unable to tolerate any dose of LPS. Recombinant mouse IL22 (rIL22, BioLegend, San Diego, CA, 10 μg/animal) was injected intravenously (IV) before IU LPS treatment and 24 h after. During IU LPS treatment procedure, the number of implantation sites was recorded. The timing of preterm delivery and number of live pups were assessed during harvest, 48 h post IU LPS treatment. For tissue harvests, a separate group of animals were euthanized 6 h after surgery. The injected/right uterine horn was incised longitudinally along the antimesenteric border. Gestational tissues, mesometrial decidua, and junction zones or placenta were harvested and flash-frozen in liquid nitrogen and stored at –80°C for mRNA and protein extraction. Whole implantation sites with pups removed were fixed in 4% paraformaldehyde for immunohistochemistry.

CD-1 mice

Intrauterine injection of LPS (extracted from *Salmonella enterica*, L2262, Sigma, St. Louis, MO, 100 μg/animal) was performed into the right uterine horn on day 14.5 of a 19–20-day gestation period, as previously described [9, 40, 12]. This intervention reliably induced preterm delivery within 18–24 h. Recombinant mouse IL22 (rIL22, BioLegend, San Diego, CA, 10 μg/animal) was injected IV 3 h after IU LPS. The timing of preterm delivery and number of live pups delivered at term were observed.

Immunofluorescence and immunohistochemistry

For the detection of uterine NK cells DBA-lectin staining was done using standard procedures [29, 30, 42]. Fixed-frozen implantation site tissue sections (5 μm) were blocked with an Avidin/Biotin Blocking Kit (Vector Labs Cat# SP-2001). Following blocking sections were incubated with biotin-conjugated DBA-lectin (Vector Labs Cat# B-1035) (1 μg/ml in 3% phosphate buffered saline [PBS] supplemented with 3% bovine serum albumin [BSA]) overnight at 4°C, followed by incubation for 1 h at RT with Avidin-conjugated Texas Red (Vector Labs Cat# A-2006). All antibodies used in the study are listed in Supplementary Table 1. Negative control sections were similar treatments that did not contain biotin-conjugated DBA-lectin. For other immunofluorescence staining, sections were incubated with mouse FITC-anti-Pan Cytokeratin (Sigma-Aldrich Cat# F0397), rabbit anti-interleukin 22 receptor, alpha 1 (IL22RA1) (Millipore Cat# 06-1077), rat anti-IL22 (R&D Cat# MAB582) or isotype-matched controls, overnight at 4°C. Primary antibodies were followed by secondary antibodies, FITC-rabbit anti-rat (Vector Labs Cat# FI-4000), FITC-goat anti-rabbit (Vector Labs Cat# FI-1000), or Alexa-647-goat anti-rabbit (Invitrogen Cat# A21246). Cells were fixed in Prolong gold antifade reagent with DAPI (Invitrogen Cat# P36934) to visualize nuclei. Antigen distribution was examined using a Nikon Eclipse TE2000-S fluorescence microscope (Nikon Instrument INC).

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

Total RNA from either the mesometrial decidua and junction zone or the placental tissues were extracted. Homogenization was performed in either Ambion TRIzol Reagent (Life Technologies Cat# 15-596-018) or using an RNasy Micro Kit (Qiagen Cat# 74004) according to the manufacturer's protocol. For ex vivo experiments, cell suspensions were collected, centrifuged; the medium was separated and the pellet was lysed in Trizol. Complementary DNA was prepared using TaqMan Fast Advanced Master Mix (Applied Biosystems Cat# 4444557). Duplex RT-PCR was performed with one primer pair amplifying the gene of interest and the other an internal reference (*Actb* or *Gapdh*) in the same tube using the Applied Biosystems Step One Real-time PCR system. Semiquantitative analysis of gene expression was done using the comparative CT ($\Delta\Delta$CT) method, normalizing expression of the gene of interest to actin, beta (*Actb*) or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The prevalidated Taqman gene expression assays for *Il22* (Mm01226722_g1), *Il22*ra1 (Mm01192943_m1), *Ifng* (Mm01168134_m1), T*nf* (Mm00443258_m1), chemokine (C-C motif) ligand 3 (*Ccl3*) (Mm00438260_s1), BCL2-associated X protein (*Bax*) (Mm00432051_m1), B cell leukemia/lymphoma 2 (*Bcl2*) (Mm0047763_m1) (ThermoFisher Scientific), and internal control *Actb* (Mm02619580_g1) (ThermoFisher Scientific) or *Gapdh* (4352339E) (Applied Biosystems) were used. Real-time PCR was performed using the universal PCR master mix reagent (Applied Biosystems).

Placental cells preparation and ex vivo treatment

Uteri were dissected on day 14.5 of pregnancy, and placentas were dissected from decidual caps; a single-cell suspension was prepared as described previously [43]. Briefly, tissues were minced in Hanks balanced salt solution (Life Technologies), mechanically dispersed through a 100-μm nylon filter, and centrifuged at 1500 rpm. The remaining pellet was dispersed in Roswell Park Memorial Institute (RPMI) medium at 10^7cells/ml in 48-well plates. Prior to plating, placental suspensions underwent red cell lysis by incubation with red blood cell lysis buffer (BioLegend). The above specimens were incubated at 37°C in 5% CO_2/95% air for 1 h. Viability of ex vivo cultured cells was >95% as assessed using the trypan blue dye exclusion test. Placental cells underwent treatment for 2 h with PBS or LPS (5 ng/ml) followed by supplementation with rIL22 (5 μg/ml) for an additional 12 h.

Protein extraction

For protein extraction, cells were homogenized in ice-cold 1X radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology) containing protease and phosphatase inhibitors (Roche Applied Science). Lysates were incubated on ice for 30 min and centrifuged at 10 000× g for 10 min at 4°C. Supernatant fluid was collected and used as a total cell lysate for protein assays. Protein concentration was measured by bicinchoninic acid (BCA) protein assay.

Caspase activity and fas ligand (FASL) measurement

Activity of Caspase9, -8, -3, and -1 (CASP9, -8, -3, -1) was measured using SensoLyte AFC Caspase Profiling Kit (AnaSpec, Fremont, CA) [44] in total protein extracted from decidual cells and placental cells treated ex vivo with LPS, with or without rIL22. Caspase activity was assayed on a fluorometer (Bio-Tek Instruments) as per the instructions provided by manufacture. Equal amounts of protein (50 μg) were used. Caspase activity was measured in relative fluorescence units/μg of protein. FASL was analyzed by Milliplex map kit (Millipore, St. Charles, MO) according to the manufacturer's instruction.

Statistical analysis

The data are expressed as mean ± SEM. The statistical analyses were performed using MedCalc software (version 13.0.4.0). Data were assessed with one-way or two-way analysis of variance (ANOVA). When the data were not normally distributed, two groups were

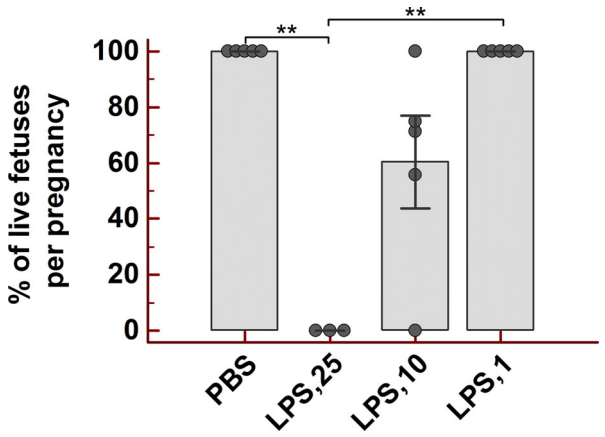

Figure 1. Intrauterine LPS administration in C57BL6 mice reliably induces preterm birth with the 25 μg/mouse dose, while the 1 μg/mouse dose is well tolerated. LPS was injected into the uterus on gd 14.5 (LPS dose of 1ug (n = 5), 10ug (n = 5), 25ug (n = 3), PBS (n = 5)), and the uterus was collected 48 h post-treatment. The number of live or dead fetuses and aborted implantation sites was documented. Data analyzed by ANOVA followed by Tukey–Kramer post hoc analysis, $^{**}P < 0.01$.

compared with the Kruskal–Wallis test. $P < 0.05$ was considered significant.

Results

Intrauterine lipopolysaccharide administration induces uterine interleukin 22 expression

To reveal if the expression of IL22 is affected during preterm birth, we used a well-defined mouse model that is based on IU LPS administration to a prepartum mouse [12]. C57BL6 mice (referred further as WT) were treated with different doses of LPS on gestation day (gd) 14.5 to determine the dose that reliably induces abortion in this strain within 48 h after the injection. The administration of LPS 25 μg/mouse caused complete loss of fetuses by the time of tissue collection either due to preterm delivery or IU death (Figure 1). In contrast, the LPS dose of 1 μg/mouse was tolerated well, suggesting that protective mechanisms were effective in prevention of preterm delivery.

The expression of IL22 was analyzed in uterine samples collected 6 h after the administration of either 25 or 1 μg of LPS and compared to control samples from animals that received IU infusions of PBS. None of the control samples revealed the presence of *Il22* transcripts, but we found the expression of *Il22* in all LPS-treated animals (Figure 2A). Interestingly, the animals administered with low-dose LPS (1 ug/mouse) revealed the highest *Il22* levels.

The LPS administration also caused increased uterine expression of *Ifng* (Figure 2A). Other proinflammatory cytokines that were analyzed in uterine tissues demonstrated either no change (*Tnf*) or significant decrease (*Ccl3*) (Figure 2A). To check the systemic response to IU LPS administration, spleen samples were analyzed similarly (Figure 2B). No significant difference was found in mRNA levels of Ifng, *Tnf*, or *Ccl3* in the spleen of LPS-treated animals compared to PBS-treated controls. Interestingly, a substantial expression of *Il22* was observed in the spleen of animals that received a high dose of LPS (25 ug/mouse). *Il22* was also detected in the spleen of animals treated with 1 μg LPS, but not in the spleen of control animals.

To determine the IL22 source at the maternal–fetal interface, implantation sites were stained with DBA-lectin, which reacts with uterine NK cells, and with anti-IL22. DBA-lectin-positive cells were localized in decidua (i.e., maternal tissues, which will be referred to as uterine tissues), but not in placenta (a fetal tissue). Demarcation of different zones within an implantation site is shown in Figure 3A,

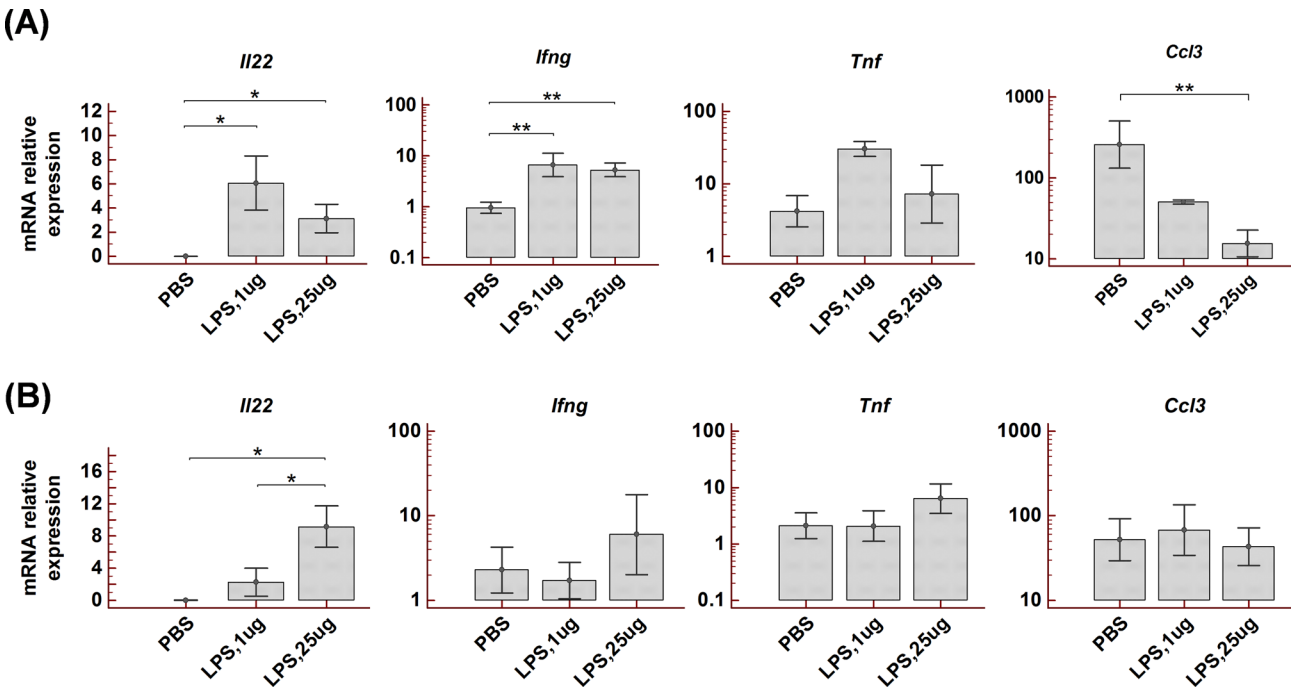

Figure 2. Effect of IU LPS administration to C57BL6 mice on gd 14.5 on cytokine expression in uterine tissue and spleen. (A) Expression of *Il22*, *Ifng*, *Tnf*, and *Ccl3* mRNA in the uterine samples collected 6 h post-LPS injection. (B) mRNA expression of these cytokines in spleen collected 6 h post-LPS injection. Animals administered an LPS dose of 1 μg (n = 5) or 25 μg (n = 5) per mouse were compared with mice receiving PBS (n = 5). Data presented as Mean ± SEM. Data analyzed by Kruskal–Wallis test or ANOVA followed by Tukey–Kramer post hoc analysis, $^{*}P < 0.05$, $^{**}P < 0.01$.

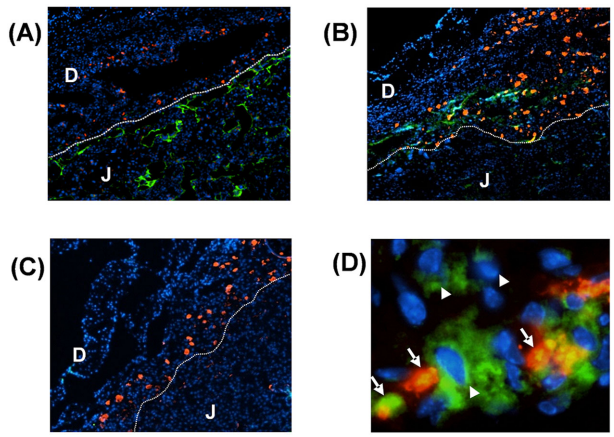

Figure 3. Intrauterine LPS administration in mice on gd 14.5 induces the expression of IL22 at the maternal–fetal interface. (A) Red—DBA lectin, green—pan-cytokeratin, blue—DAPI staining for nuclei. Line is drawn to indicate demarcation of different zones within the maternal–fetal interface. Decidua can be identified by presence of DBA-positive uNK cells. Junctional zone of the placenta is identified by pan-cytokeratin-positive trophoblasts. Original magnification 4×. (B, C, D) Red—DBA lectin, green—IL22, blue—DAPI. (B) IL22 staining is observed in samples from mice treated with 25 μg/mouse LPS. IL22 expression is localized in decidua along with DBA-lectin (red) positive uNK cells in areas adjacent to the junctional zone. Original magnification 4×. (C) No IL22 staining was detected in samples from control PBS-treated mice. Original magnification 4×. (D) A representative image of IL22 expression in decidua from LPS-treated mouse. Both DBA-lectin positive uNK cells (arrow) and DBA-lectin negative cells (arrow head) are found to express IL22. Original magnification 40×. D—decidua, J- junctional zone of placenta.

where the junctional zone can be distinguished by bright cytokeratin staining specific for trophoblasts and the decidual area by DBA-lectin staining characteristic of uterine NK cells. Samples from LPS-treated animals but not control PBS-treated animals revealed positive IL22 staining (Figure 3B and D). The IL22 staining was observed in decidua in areas adjacent to large blood vessels and to the junctional zone of placenta and was either colocalized with DBA-lectin staining or not (Figure 3D). Also, some weak but persistent diffuse staining of IL22 was observed within the junctional zone.

Interleukin 22-deficient mice are highly susceptible to lipopolysaccharide-induced preterm birth

To investigate whether the observed uterine IL22 increase after LPS challenge is a part of the pathogenic mechanism that leads to pregnancy loss or if it is an important factor in preterm birth prevention, mice deficient for IL22 were tested with IU LPS administration. *Il22-/-* mice were found to be very sensitive to LPS treatment as indicated by the dose response data (Figure 4A). The large dose of LPS (25 μg/mouse) caused complete abortion of fetuses within the first 24 h, and the uterus was significantly reduced in size by the time of tissue collection (Figure 4B). However, in contrast to WT mice, the LPS dose of 1 μg/mouse was not tolerated by *Il22-/-* mice and caused complete pregnancy loss. Moreover, the LPS dose of 0.1 μg/mouse was also efficient in induction of preterm birth in *Il22-/-* mice. A comparison of cytokine expression in uterine tissues from PBS- or LPS-treated animals did not reveal a significant difference for *Ifng* or *Ccl3*, although significantly higher *Tnf* expression was detected in mice administered with LPS (Figure 4C).

Recombinant interleukin 22 reduces the risk of lipopolysaccharide-triggered abortion and intrauterine fetal death

To explore IL22 as a therapeutic agent for the prevention of LPS-induced preterm labor, the *Il22-/-* mice were treated with two doses of rIL22, 10 μg each, injected IV before and 24 h after LPS administration. As shown in Figure 4B and D, IL22 supplementation in animals that received 1 μg or 0.1 μg/mouse of LPS significantly increased the number of live fetuses or prevented preterm labor compared to animals that received the same LPS treatment alone. To our knowledge, this is the first report to demonstrate that IL22 is a potential mediator to prevent LPS-induced preterm delivery. Subsequent experiments with rIL22 supplementation were performed using CD1 mice. Compared to inbred C57BL/6J strain, the CD-1 is an outbred strain with much higher fecundity. Therefore, use of this strain expedites reaching the required sample size. Intrauterine 100 μg/mouse LPS administration is known to reliably induce preterm birth in this strain [12, 40]. As shown in Table 1, IL22 supplementation prevented LPS-induced preterm labor by ∼78% and significantly increased the number of live pups delivered at term compared to animals without supplementation.

Interleukin 22 target cells are located within junctional and labyrinth zones of placenta

IL22 acts via the IL22 receptor, which is a heterodimer consisting of unique alpha subunit, IL22RA1, and a common IL10 beta subunit, IL10RB. We evaluated *Il22ra1* mRNA expression in uterine samples from WT and *Il22-/-* animals treated with LPS or PBS (Figure 5A). *Il22*ra1 levels in WT as well as *Il22-/-* mice were not affected by LPS treatment. However, it was found that *Il22*-deficient mice, despite the absence of *Il22*, demonstrated significantly higher *Il22*ra1 expression in comparison to WT mice. Immunofluorescent staining was performed to investigate IL22RA1 expression within the implantation site. Cells stained positive for IL22RA1 were mainly distributed in the junctional zone of placenta consisting of various cytokeratin positive trophoblast populations (Figure 5B). Moreover, IL22RA1 expression was observed throughout the labyrinth zone of the placenta (Figure 5C).

rIL22 supplementation prevents activation of the extrinsic pathway of apoptosis in placental cells

To further assess the mechanism of the observed protective effect of IL22, we characterized the intrinsic and extrinsic pathways of apoptosis [44, 45] in ex vivo cultured placental cells incubated with LPS or PBS for 2 h, followed by treatment with or without rIL22 for 12 h. The factors involved in the intrinsic pathway of apoptosis (BAX, BCL2, and CASP9) remained unchanged in placental cells (Figure 6A). However, supplementation with rIL22 significantly reduced the LPS-induced level of proteins involved in the extrinsic pathway of apoptosis (CASP8 and CASP3) (Figure 6B). Therefore, the data suggest that IL22 prevents LPS-induced activation of the extrinsic pathway of apoptosis at the feto–maternal interface.

Discussion

We have identified the important role of IL22 in controlling the uterine reaction to bacterial endotoxin exposure during late pregnancy in mice. IL22 is produced by decidual cells in response to IU LPS administration but primarily targets placental cells. Our data suggest that IL22 prevents preterm birth and IU fetal death and promotes

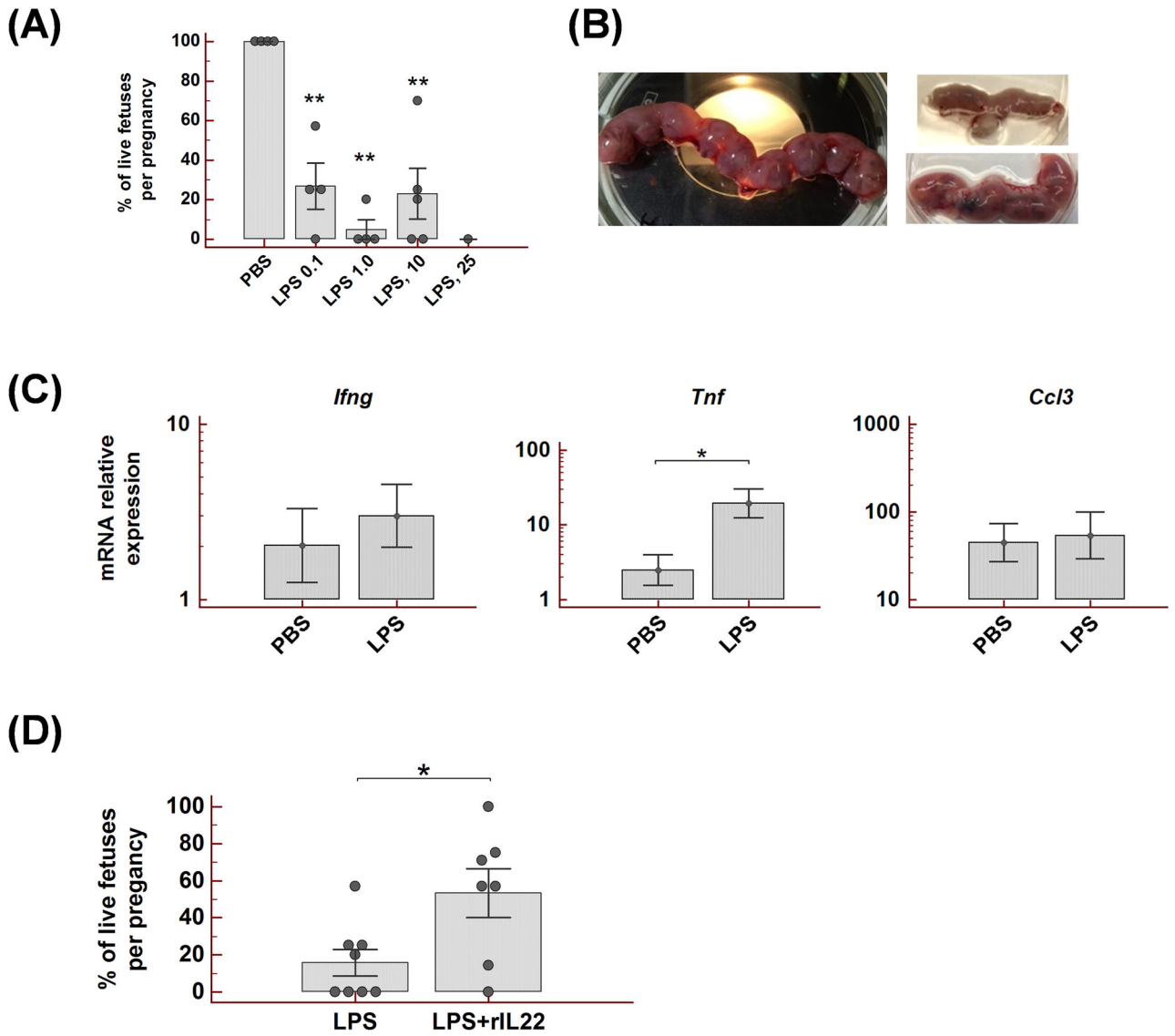

Figure 4. IL22-deficient mice are highly susceptible to LPS-induced preterm birth. The risk of LPS-triggered abortion and IU fetal death is reduced by rIL22 supplementation. (A) Pregnant *Il22*-/- mice received IU injection of different doses of LPS (25 μg (n = 1), 10 μg (n = 5), 1 μg (n = 4), or 0.1 μg/mouse (n = 4)) or PBS (n = 4) on gd 14.5. The uterus was collected 48 h post-treatment and the number of live or dead fetuses and aborted implantation sites was documented. Percent of live fetuses per pregnancy in LPS-treated animals was compared to PBS-treated mice. (B) Images of uteri from *Il22*-/- mice collected 48 h post-LPS or -PBS injection. Left image: uterus from PBS-treated mouse. Right upper: uterus from mouse treated with 1 μg LPS. Right lower: uterus from mouse treated with 1 μg LPS along with rIL22 supplementation. (C) Expression of *Ifng, Tnf,* and *Ccl3* mRNA in the uterine samples from *Il22*-/- mice. Uteri were collected 6 h post-LPS (1 μg, n = 5) or PBS injection (n = 4). (D) Pregnant (gd14.5) *Il22*-/-mice received IU injection of LPS (1 or 0.1 μg) with or without rIL22 supplementation. Percent of live fetuses per pregnancy was calculated 48 h post-treatment. N = 8 in LPS group, n = 7 in LPS + rIL22 group. Data analyzed by Kruskal–Wallis test or ANOVA followed by Tukey–Kramer post-hoc analysis, $^{*}P < 0.05$, $^{**}P < 0.01$.

survival of placental cells. IL22 is a cytokine expressed by various immune cells but targets primarily epithelial and stromal cells [36]. Cells with potential to secrete IL22 were described both in mouse and human endometrium. In human early pregnancy decidua, the ILC3 were shown to release IL22 [33]. The authors demonstrated that these ILC3 could partially differentiate into uNK cells. In mouse, the DBA-lectin-positive uNK cells, which constitute a major lymphocyte population during midgestation, were reported as potential IL22 producers [30]. The ILC3 are also present in the murine uterus where they are sparsely distributed in the myometrial layer of uterus [32, 46].

While there was no detectable IL22 in gd 14.5 uterine tissues from control mice, we found IL22 in all uterine samples from LPS challenged mice. IL22 staining within the utero-placental unit demonstrated that IL22 is secreted by cells distributed along the junctional zone of the placenta. In addition, some weak diffuse IL22 staining was observed within the spongiotrophoblast/junctional zone, which could be the secreted form and not cell-associated IL22. Simultaneous staining with anti-IL22 Abs and DBA-lectin revealed that besides DBA-lectin-positive uNK cells, IL22 is also produced by DBA-lectin negative cells as well. Those might include NK cells, ILC, T cells, or myeloid cells [30, 32, 47, 48]. The source of the IL22 in the

Table 1. IL22 supplementation inhibits LPS-induced preterm delivery.

Treatment groups dose/mouse	Preterm delivery (%)	Number of pups delivered at term
LPS IU (100 μg) + PBS IV (100 μl)	8/8 (100)	0
LPS IU (100 μg) + rIL-22 IV (10 μg)	2/9 (22)**	9.57 ± 1.13**,¶
PBS IU (100 μl) + rIL-22 IV (10 μg)	0/3 (0)	12.02 ± 1.23
PBS IU + PBS IV (100 μl)	0/3 (0)	12.14 ± 1.14

**$P \leq 0.01$, LPS + PBS vs LPS + rIL22.
¶Includes only pups from pregnancies delivered at term.
IV injections performed 3 h after IU inoculation.

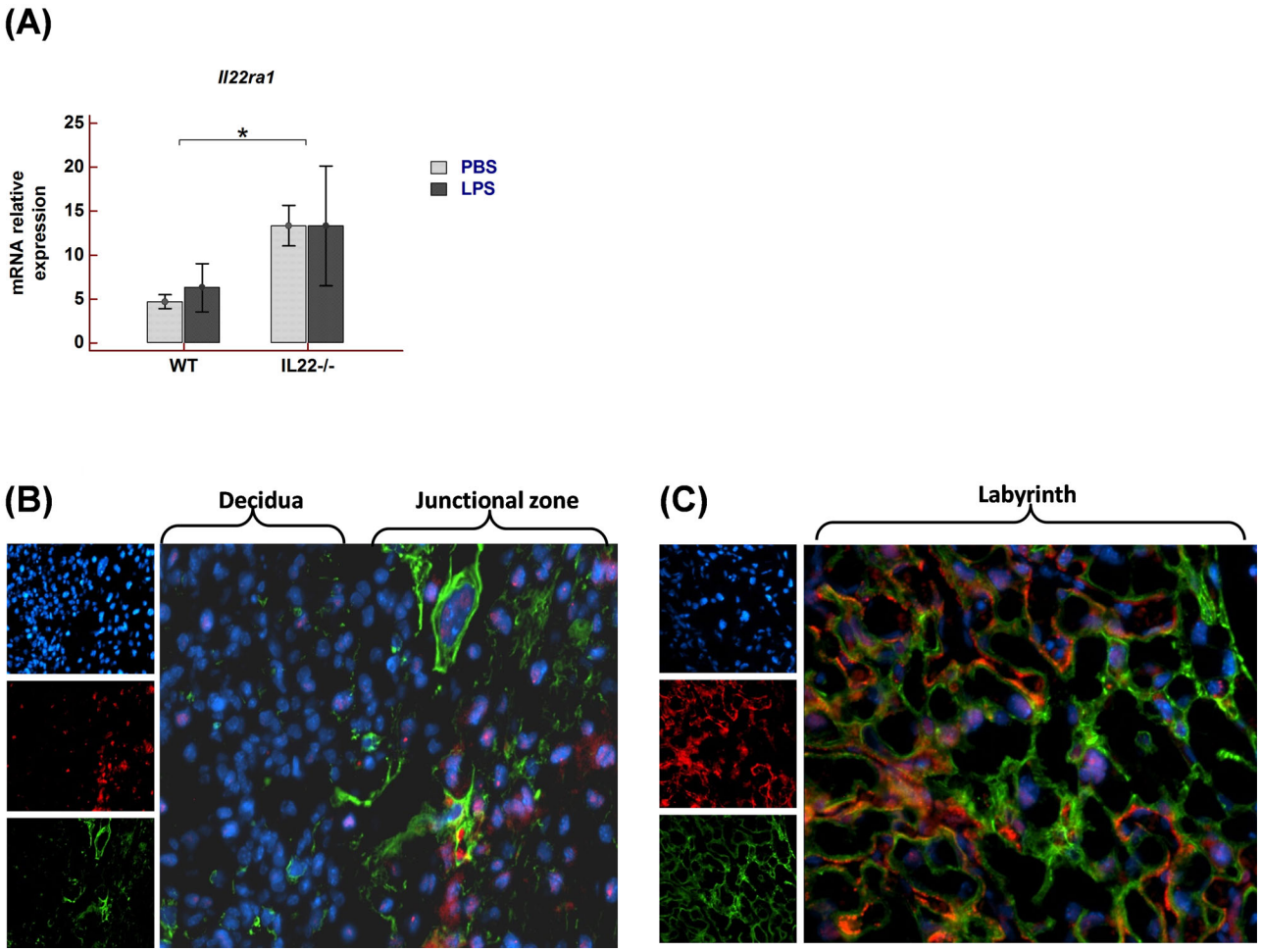

Figure 5. IL22RA1 expression in murine implantation site. (A) *Il22*ra1 mRNA expression in uterine samples from pregnant WT and *Il22*-/- mice. Mice were treated with PBS (n = 5 for WT, n = 4 for *Il22*-/-) or LPS dose that reliably induces preterm birth (25 μg for WT (n = 5) and 1 μg (n = 5) for *Il22*-/- mice) and tissues were collected 6 h post LPS treatment. Data presented as Mean ± SEM. Data analyzed by two-way ANOVA with Bonferroni correction, *$P < 0.05$. (B) and (C) Immunofluorescent staining with antibodies against IL22RA1 (red) and pan-cytokeratin (green) in gd14.5 implantation site sections from WT mice. Small panels: upper—DAPI (blue) for nuclei, middle—IL22RA1, bottom—pan-cytokeratin; large panels: merge. Decidua and junctional zone of placenta are shown in (B), placental labyrinth is shown in (C). Original magnification 20×.

decidua should be characterized. For instance, a secretion of IL22 upon LPS stimulation of bone marrow-derived DC was described [48]. Because myeloid cells present in high numbers in murine uterus during pregnancy, they indeed can be explored as potential IL22 producers.

In our studies, the LPS-induced upregulation of *Il22* in uterine tissues collected 6 h postsurgery was accompanied by increased expression of *Ifng* and *Tnf*, although for *Tnf* the difference was not statistically significant. Interestingly, a similar relatively low response was seen with a leukocyte influx to the uterus assessed shortly after IU LPS challenge in pregnant gd 16 mice [22]. The analysis at 7 h post-LPS administration revealed a marked leukocyte infiltration into the lungs and liver but little into the uterus; the increase in a number of mobilized leukocytes was observed only at the onset of parturition. Further, Edey et al. [22] demonstrated significantly higher numbers of infiltrating inflammatory monocytes and neutrophils at 7 h post-surgery in the uterus of control PBS-treated animals when compared to LPS-treated mice. These data are consistent with our data for

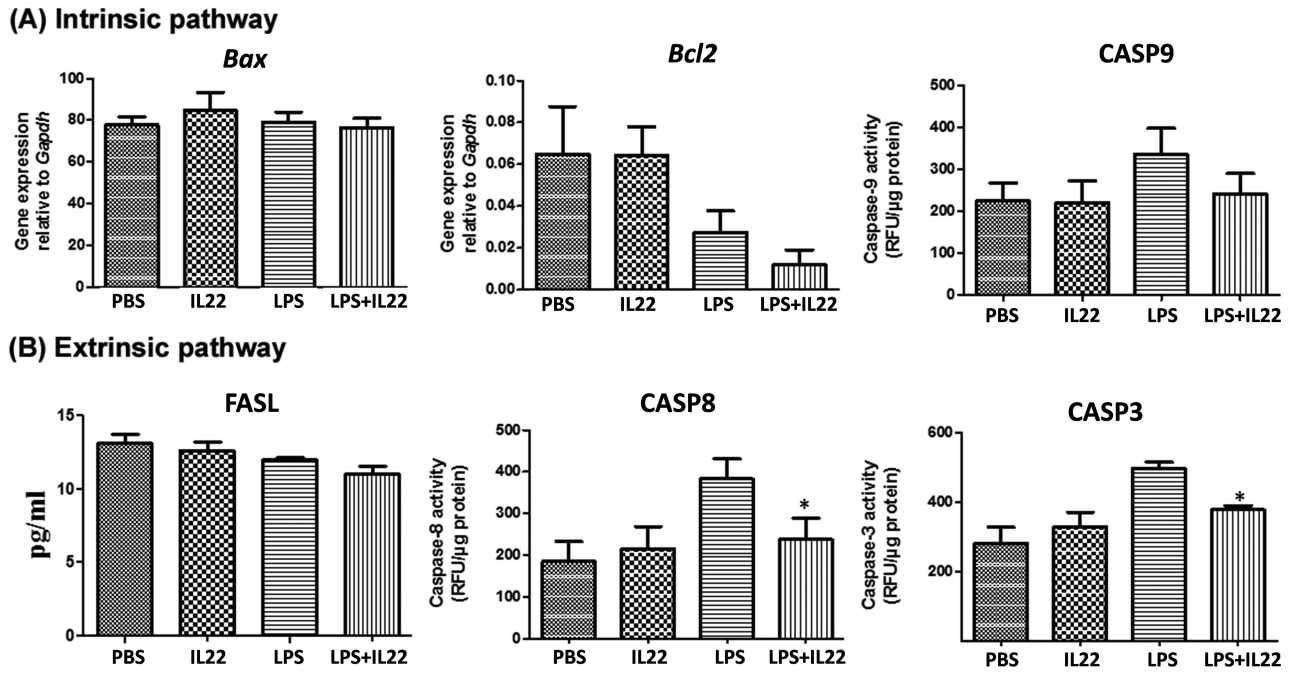

Figure 6. rIL22 supplementation prevents activation of extrinsic pathway of apoptosis in placental cells. mRNA expression of *Bax* and *Bcl2*, and activity of CASP9, molecules involved in the intrinsic pathway of apoptosis (A) and, protein concentration of FASL and activity of CASP8 and CASP3, molecules involved in extrinsic pathway of apoptosis (B) in placental cells cultured ex vivo with PBS (control) or LPS for 2 h, followed by treatment with rIL22 or control for 12 h. Depicted are representative figures from three repeat experiments. Data presented as Mean ± SEM. Data analyzed by ANOVA followed by Tukey–Kramer post hoc analysis. $^{*}P < 0.05$ compared to LPS with and without IL22.

higher *Ccl3* levels in uterus of PBS-treated animals than in animals treated with LPS, as CCL3 is the main chemokine for neutrophil trafficking. Upregulation of uterine *Il22* expression was also reported in response to systemic LPS challenge (intraperitoneal administration) in pregnant gd 8.5 mice [49]. Within the first 8 h post-LPS treatment, a ten-fold increase of *Il22* transcript expression was found, while 16 h post-LPS treatment there was no upregulation of *Il22*. Thus, it seems that *Il22* upregulation in the uterus of pregnant mice is an element of the body's early response to an inflammatory agent, like bacterial LPS. In addition to uterine upregulation of *Il22*, a significant increase in expression of *Il22* was observed in the spleen. The systemic IL22 response was also reported after intraperitoneal LPS challenge [50]. Th17 cells or a discrete innate lymphoid population with CD25 + CCR6+ phenotype could be considered among cellular sources of IL22 in the spleen [51]. IL22 is known as a cytokine with the properties of a double-edged sword cytokine, capable of mediating both inflammatory and anti-inflammatory responses (reviewed in [36, 52]). *Il22-/-* mice were tested by IU LPS administration to investigate whether the observed uterine *Il22* upregulation in response to LPS challenge is a part of the pathogenic mechanism that leads to pregnancy loss or if it is an important factor in preterm birth prevention. Indeed, pregnant *Il22-/-* mice were highly susceptible to LPS treatment and responded with increased uterine *Tnf* levels. Importantly, the risk of LPS-triggered abortion and IU fetal death in *Il22-/-* mice was significantly reduced with rIL22 supplementation. Moreover, the use of rIL22 successfully inhibited LPS-induced preterm birth in normal CD1 mice. Interestingly, LPS challenge of *Il22-/-* mice did not upregulate uterine *Ifng* expression nor modified *Ccl3* transcripts level. However, when the cytokine response was compared between LPS-treated *Il22-/-* and WT mice, no significant differences between two groups were found. In contrast, LPS treatment in *Il10-/-* mice was reported to cause a dramatic increase in proinflammatory cytokines when compared to WT mice [13]. These results suggest different mechanisms of preterm birth in *Il10-/-* and *Il22-/-* mice.

Duffin et al. 2016 [50] demonstrated that rIL22 treatment reduces the risk of systemic inflammation in mice injected with LPS intraperitoneally. The authors determined that IL22 production by intestinal ILC3 is essential in protecting against gut barrier dysfunction to prevent systemic inflammation. LPS-induced PGE2 was shown to directly activate intestinal ILC3 and induce IL22 secretion. IL22 targets intestinal mucosal epithelium and promotes its survival and regeneration [37, 50].

With regard to the IL22 receptor, previously it was reported that *Il22*ra1 mRNA in mice was detected at very low levels in restricted organs (such as the kidney, liver, lung), but was upregulated upon LPS stimulation [53]. Our data demonstrate that expression of IL22RA1 in murine late gestation uterus does not require LPS stimulation. Immunofluorescent staining revealed similar staining in control and LPS-treated mice that was mainly localized to placental cells, namely cells in the junctional zone and the labyrinth. Higher expression of uterine IL22RA1 was detected in *Il22-/-* mice when compared to WT mice, possibly as a mechanism to account for a lack of IL22. Human trophoblast cells were also reported to express IL22RA1 [35]. It was shown that IL22 promotes survival of trophoblasts, while reduced secretion of IL22 by decidual NK cells affects trophoblast growth and is involved in the occurrence of spontaneous miscarriage [35]. Our results suggest that IL22 supplementation is important to prevent LPS-induced apoptosis in trophoblasts.

Some limitations of our findings include relatively small sample size of the experimental groups and not a completely defined cellular

source of IL22 at the implantation site. Further experiments with LPS challenge at different gestation time-points, e.g., gd 7.5–10.5, when DBA-lectin-positive uNK cells were the most abundant, will be informative to determine IL22-producing cells in murine uterus.

In conclusion, our study provides evidence that IL22 plays an important role in controlling the risk of inflammation-induced preterm birth. The upregulation of IL22 in response to LPS challenge contributes to defense against inflammatory responses at the maternal–fetal interface and prevents apoptosis of placental cells. This study features IL22 as a new approach to treat and prevent preterm birth in patients at risk.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table S1. Antibodies used in the study.

Acknowledgments

Author contributions: SD, SS, and MKJ designed and performed research, analyzed data, and wrote the paper. VA and GKK performed research, AGS helped in writing the paper. EH and KDB designed research, analyzed data, and helped in writing the paper.

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