ARTICLE

Relevance of placental type I interferon beta regulation for pregnancy success

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Pregnancy is a unique immunologic and microbial condition that requires an adequate level of awareness to provide a fast and protective response against pathogens as well as to maintain a state of tolerance to paternal antigens. Dysregulation of inflammatory pathways in the placenta triggered by pathogens is one of the main factors responsible for pregnancy complications. Type I IFNs are key molecules modulating immune responses at the level of the placenta and are crucial for protection of the pregnancy via their antiviral and immune modulatory properties. In this study, we elucidate the mechanisms controlling the basal expression of IFNβ and its negative feedback. Using in vitro and in vivo animal models, we found that TLR signaling maintains basal IFNβ levels through the TLR4-MyD88-independent TBK/IRF3 signaling pathway. We describe the role of the TAM receptor Axl in the regulation of IFNβ function in human and mouse trophoblast cells. The absence of TAM receptors in vivo is associated with fetal demise due to dysregulation of IFNβ expression and its pro-apoptotic downstream effectors. Collectively, our data describe a feedback signaling pathway controlling the expression and function of IFNβ in the trophoblast that is essential for an effective response during viral and microbial infections.

Keywords: trophoblast; immune regulation; cytokine; TAM receptors; type I interferon; Interferon beta; ISGs

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INTRODUCTION

An effective immune response against pathogens requires an adequate level of awareness to provide a rapid and protective response as well as mechanisms that will restrict the degree of the response to prevent a chronic inflammatory stage that can cause tissue damage and disease.[1,2]

Microorganisms and their products are sensed by immune sensors such as Toll-Like receptors (TLRs).[3] In the case of lipopolysaccharide (LPS), the classical response involves the TLR4/MyD88/nuclear factor-kappa-B (NF-κB) pathway leading to the production of chemokines [e.g., interleukin (IL)-8 and C-C motif ligand 2] and cytokines (e.g., IL-1β and tumor necrosis factor-α (TNFα)).[3] A second major group of cytokines produced by LPS signals through TLR4 are the type I interferons (IFNs) alpha and beta, mediated by the MyD88-independent pathway via TRIF and IFN regulatory factor 3 (IRF3).[4] Activation of the classical MyD88-dependent or alternative MyD88-independent pathway depends on the cell type or LPS concentration. Furthermore, the induction of type I IFN through TLR4 suggests cross talk with other TLR pathways, such as TLR3, which are known to be the main inducers of type I IFNs.

Pregnancy is a unique immunologic and microbial condition in which the cells at the maternal–fetal interface and microbial products contribute to establishing a delicate balance between protecting the fetus against dangerous signals and inducing specific tolerance to paternal antigens.[5] Disruption of the balance between protection and tolerance may lead to pregnancy complications such as recurrent pregnancy loss, fetal damage, and even maternal mortality.

The trophoblast represents the first point of contact between the blastocyst and the maternal decidua and has an active role in shaping the immunological milieu at the implantation site.[5] The trophoblast has the ability to sense and respond to its microenvironment through the expression of pattern recognition receptors such as TLRs,[6–8] which can recognize specific molecular patterns in the microenvironment.

In previous studies, we demonstrated that in contrast to high concentrations of LPS, which induce pregnancy termination, the same LPS, but at lower concentrations, had no detrimental effect on pregnancy.[9] However, the non-detrimental responses observed at low concentrations of LPS were reversed due to a pre-occurring viral infection that affected the IFNβ pathway. This observation of a viral infection affecting the TLR4-LPS response was defined as the "double hit model of preterm birth", wherein a viral infection can sensitize trophoblasts to a low dose of LPS.[9] Interestingly, our findings, as well as those of others, have conclusively shown that

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IFNβ is important in the modulation of TLR4 responses to LPS during pregnancy.[10–12] However, the molecular mechanisms that mediate the interaction between LPS/TLR4 and the regulation of type I IFNβ expression at the placenta are poorly understood.

Type I IFNs (IFNα and IFNβ) are polypeptides that are able to induce an antimicrobial state, modulate innate immune responses, and induce activation of the adaptive immune system.[13,14] Type I IFN responses can be induced by both viral and bacterial pathogens that are sensed by TLRs, NOD-like receptors, and RIG-I-like receptors promoting signaling through stimulator of IFN genes (STING).[15] Constitutive, baseline expression of type I IFN is very low in most tissues and can rapidly be triggered by viral attack and bacterial infections.[16] Following secretion from cells, type I IFNs mediate their effects by binding to their cell surface receptor, type I interferon-associated receptor (IFNAR), and activating members of the JAK kinase family.[17] Activated JAK kinases phosphorylate the signal transducer and activator of transcription family of transcription factors, which homo- or heterodimerize and form complexes (GAS; ISGF3) with other transcription factors to activate the transcription of IFN-stimulated genes (ISGs). ISGs are the primary effectors of type I IFN-mediated biological responses.[13,18] Despite being identified and named for their ability to interfere with viral replication in infected cells, IFNs, through specific ISGs, have immunomodulatory, cell differentiation, anti-angiogenic, and pro-apoptotic effects.[19] However, in addition to their protective effects, type I IFNs can have deleterious effects during bacterial infections and complications such as autoimmune diseases, which have been associated with excessive or chronic type I IFN responses. Therefore, regulation of their expression and function is critical for an efficient immune response and maintenance of tissue homeostasis.

The regulation of type I IFN signaling includes factors that maintain basal or tonic expression and a regulatory mechanism that limits the magnitude and time of the response.[19] Recent studies have demonstrated that basal IFNβ production under homeostatic conditions is provided by factors from commensal bacteria through TLR4, which contributes to the modulation of the local immune responses[20] and protection of mucosal tissues against viral infections.[21]

In addition, there are mechanisms that suppress type I IFN signaling to prevent deleterious effects such as the induction of apoptosis.[22] TYRO3, AXL, and MER (TAM) receptors are major regulators of type I IFN signaling. TAM receptors are membrane tyrosine kinase receptors that are found in high abundance in immune cells and have been reported to regulate innate immune responses by dampening TLR signaling. Loss of TAM receptor signaling has been associated with stages of hypersensitive inflammatory responses implicated in sepsis, chronic inflammatory disease, and autoimmune diseases.[23,24] TAM receptor signaling has been found to inhibit TLR-induced inflammatory cytokines and, most notably, to inhibit type I IFN receptor activation.[25,26]

In the context of pregnancy, we have shown that loss of IFNβ signaling in the placenta leads to uncontrolled viral replication and fetal viral infection,[27] maternal mortality,[27] and hypersensitivity to bacterial products,[9] suggesting a critical role for IFNβ signaling in the protection of pregnancy. Although the role and regulation of type I IFN expression and function has been extensively studied in many organs and barrier surfaces, such as the skin and upper respiratory and gastrointestinal tracts, the regulatory factors responsible for IFNβ expression and function during pregnancy are still poorly understood.

In the present study, we tested the hypothesis that products from commensal bacteria (LPS) can maintain basal or tonic IFNβ expression in trophoblast cells and that the TAM receptor Axl mediates a negative feedback pathway that suppresses IFNβ expression.

The objectives of this study were to characterize the molecular pathways involved in the expression of IFNβ following TLR4 ligation in trophoblast cells and to elucidate the mechanisms that regulate IFNβ expression and function during pregnancy. Using in vitro and in vivo models, we describe for the first time a regulatory feedback signaling pathway that controls the expression and function of IFNβ in trophoblasts and establishes the regulation of type I IFNβ signaling as a central mechanism for the protection of pregnancy.

MATERIALS AND METHODS

Reagents and cell lines

The reagents and cell lines used in the experiments were as follows: recombinant human Gas6 (GS-050, R&D Systems, Minneapolis, MN); LPS isolated from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO); the telomerase-immortalized first-trimester trophoblast cell line; and Swan71 cells.[28]

Isolation of human primary trophoblast cells from first-trimester placenta

The use of patient samples was approved by Yale University's Human Research Protection Program. First-trimester placentas (7–12 weeks of gestation) were obtained from elective terminations of normal pregnancies performed at Yale-New Haven Hospital. Isolation of primary trophoblast cells was performed as previously described by.[28] Briefly, the tissue was washed with cold Hanks balanced salt solution (Gibco), minced, transferred to trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) digestion buffer, and incubated at 37 °C for 40 min on a shaker. The mixture was then passed through a nylon strainer and then layered over Lymphocyte Separation Medium (ICN Biomedicals, Inc., Aurora, OH, USA) and centrifuged at $400 \times g$ for 25 min. The cellular interface containing the trophoblast cells was collected and resuspended in D-MEM with D-valine (Caisson Labs, North Logan, UT, USA) supplemented with 10% normal human serum (Gemini Bio-Products, Woodland, CA, USA) and cultured at 37 °C/5% CO2.

Immunofluorescence staining

Six-micron-thick paraffin-embedded villous tissue was deparaffinized, rehydrated, and rinsed with phosphate-buffered saline (PBS), and antigen retrieval was performed by microwaving at 100% power for 10 min in 10 mM sodium citrate (pH 6.0). The tissue sections were washed with PBS and blocked with 3% normal donkey serum in an antibody dilution buffer consisting of PBS containing 0.1% Triton X-100. Sections were then incubated overnight in primary antibody (mouse anti-human Axl monoclonal antibody (ab89224, 1:200 dilutions, Abcam, Cambridge, MA) or rabbit anti-human MERTK monoclonal antibody (ab52968, 1:200 dilutions, Abcam) at 4 °C and labeled with a fluorescein-conjugated secondary antibody (Molecular Probes, Eugene, OR). Nuclei were counterstained blue with 4′,6-diamidino-2-phenylindole. Samples were observed with a fluorescence microscope (Olympus).

Western blotting

Western blot (WB) analysis was performed as previously described.[29] Briefly, cells were homogenized in phospho-cell lysis buffer (Cell Signaling, Danvers, MA), and total protein concentrations were quantified using the bicinchoninic acid assay (Pierce, Rockford, IL). Twenty micrograms of total protein was dissolved in 1× sample buffer, boiled for 5 min, and separated on a 10% SDS-polyacrylamide gel electrophoresis gel. The proteins were then transferred to polyvinylidene fluoride membranes (PerkinElmer, Waltham, MA). Membranes were blocked with 5% nonfat milk (Fisher Scientific, Pittsburgh, PA) and incubated overnight with primary antibodies in 2% nonfat milk at 4 °C. Membranes were then washed and incubated with the appropriate horseradish

Table 1. Primers used for qRT-PCR analyses (5′ → 3′)

Genes	Forward	Reverse
Human		
Axl	CCGTGGACCTACTTGGCT	CTTGGGTTATGGGCTTC
Mer	CTCTGGCGTAGAGCTATCACT	AGGCTGGGTTGGTGAAACA
GAS6	GGCAGACAATCTCTGTTGAGG	GACAGCATCCCTGTTGACCTT
IFNβ	AGCTGAAGCAGTTCCAGAAG	AGTCTCATTCCAGCCAGTGC
IFNα	GTGAGGAAATACTTCCAAAGAATCAC	TCTCATGATTTCTGCTCTGACAA
GAPDH	TGACGCTGGGGCTGGCATTG	GGCTGGTGGTCCAGGGGTCT
Murine		
IFNβ	GCACTGGGTGGAATGAGACT	AGTGGAGAGCAGTTGAGGAC
ISG20	AGCCGAFAGTGGAAACAGA	CTCGGGTCGGATGTACTTGT
TRAIL	CCTCTCGGAAAGGGCATTC	TCCTGCTCGATGACCAGCT
FAS	ATGCACACTCTGCGATGAAG	CAGTGTTCACAGCCAGGAGA
β-actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTA ACAGTCCGCCTAG

peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) at room temperature for 2 h. Membranes were washed and incubated with Western Lighting Plus (PerkinElmer) to visualize the proteins. Antibodies were diluted as follows: 1:200 anti-Axl (AF154, R&D Systems); 1:400 anti-Mer (AF891, R&D Systems); 1:1000 anti-tTBK, 1:500 anti-tIRF3, 1:1000 anti-pIRF3, and 1:1000 anti-tIRF7 (Cell Signaling); and 1:10 000 β-actin (Sigma, St Louis, MO).

RNA extraction, quantitative real-time polymerase chain reaction analysis, and ISGs (IRF7, CXCL10, ISG20, IP-10, SOCS1)
RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). RNA concentration and purity were assessed using spectrophotometric analyses of 260/280 ratios, and only samples with values of 1.8 or above were used for analysis. For quantitative analysis of messenger RNA (mRNA), 1 µg of RNA from each sample was reverse-transcribed using ISCript reverse transcription Supermix (Bio-Rad Laboratories, Hercules CA). The complimentary DNA was diluted to 1:5 in nuclease-free water, and 5 µL was used in the polymerase chain reaction (PCR) reaction. iTaq Universal SYBR green Supermix (Bio-Rad Laboratories) and gene-specific primers were added to the Reverse Transcriptase reactions and run on the CFX96, C1000 system quantitative (qPCR) machine (Bio-Rad Laboratories). The primer pair sequences are listed in Table 1. The presented values were normalized to either human GAPDH or mouse β-actin and calculated using the ΔΔCt method (ΔCt = ΔCt treated − ΔCt control), and the results are expressed as the fold change compared with the control (NT) or wild type (WT).

Cytokine and Gas6 analysis
Evaluations of cytokines and chemokines in tissues and supernatants were performed using the cytokine multiplex assay from Bio-Rad Laboratories (Luminex) as previously described.[30] The Gas6 level in supernatant was determined using the Simple Plex immunoassay system (ELLA, Protein Simple, San Jose, CA) as previously described.[31] Briefly, 50 µL of sample was added to sample inlet ports on a cartridge, and each sample from a single sample inlet port was split into multiple parallel channels. Each channel was specific for a particular analyte and subjected to a typical sandwich immunoassay protocol. For this study, we used a four-panel Simple Plex cartridge. Subsequently, the entire immunoassay procedure (sample incubation, washing, addition of secondary antibodies, washing, addition of a streptavidin tag, washing, and scanning) was performed using the software without any manual input. The analyzed results (pg/mL) were obtained using manufacturer-encoded calibration curves.

IFNβ enzyme-linked immunosorbent assay
Cell-free supernatant from LPS-treated or control trophoblasts was collected and stored at −80 °C. For the human IFNβ enzyme-linked immunosorbent assay (ELISA), the samples were thawed, and levels of secreted proteins were measured using the VeriKine IFN Beta ELISA kit (PBL Assay Science, Piscataway, NJ) per the manufacturer's protocol.

Caspase measurements
In vitro-treated cells or mouse placenta tissue was homogenized and protein extracted as described above. For this experiment, 10 µg protein lysate was incubated with either Caspase-Glo 8 or -3 substrate (Promega, Madison, WI) for 1 h at room temperature in the dark. Luminescence was measured on a TD20/20 luminometer, and activity was reported as relative light units.

Animals and animal-related procedures
C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The Axl and Mer-null double transgenics (*AxlMer*$^{-/-}$) were provided by Dr. Carla Rothlin (Department of Immunobiology, Yale University School of Medicine, New Haven, CT). *Trif*$^{-/-}$ and *Tlr4*$^{-/-}$ mice were provided by Dr. Li Wen (Department of Internal Medicine, Yale University School of Medicine, New Haven, CT). All animals were maintained in the Yale University School of Medicine Animal Facility under specific pathogen-free conditions with a 12:12-h light:dark cycle and handled in compliance with the Yale University policy for the care and use of laboratory animals. All animal experiments were approved by the Yale Animal Resource Committee (2016-11132).

Murine model
C57BL/6 and *AxlMer*$^{-/-}$ mice between 6 and 12 weeks of age were used for the experiments. The timing of pregnancy was determined by the presence of a vaginal plug, which was defined as day 0.5 post conception. At day E15.5, either sterile PBS or LPS was injected into the peritoneum of the pregnant mice at a concentration of 20 µg/kg. For outcome studies, mice were observed over the next 48 h, in the morning, at noon, and in the evening, for signs of pregnancy distress defined as (1) the presence of at least one intact or partial fetal tissue in the cage, (2) a pup in the lower vagina, and (3) an empty uterine horn with implantation sites on day 17.5 detected by intraocular injection of 1% Evans blue in a volume of 50 µL. For IFNβ, cytokines and ISG

study tissue were collected at 6 h post treatment followed by RNA extraction, or 21 h post treatment for caspase detection and immunohistochemistry.

Trif$^{-/-}$ and *Tlr4*$^{-/-}$ mice between 6 and 12 weeks of age were used for the experiments, and pregnancy was determined as stated above. At day E15.5, either sterile PBS or LPS was injected into the peritoneum of the pregnant mice at a concentration of 20 µg/kg. After 24 h, the mice were sacrificed, and the tissue was collected.

Polyinosinic-polycytidylic acid treatment

C57BL/6 mice between 6 and 12 weeks of age were used for the experiments. The timing of pregnancy was determined by the presence of a vaginal plug, which was defined as day 0.5 post conception. At day E15.5, either sterile PBS or vaccine grade polyinosinic-polycytidylic acid (poly(I:C); Invivogen) was injected intraperitoneally (i.p.) at a concentration of either 2.5 or 15 mg/kg. Mice were either sacrificed at 4 h post treatment for tissue collection or monitored for 24 h, and fetal outcome was determined.

Short hairpin RNA infection

Human *Axl* or control short hairpin RNA (shRNA) was purchased from Transomic technologies (TLHSU1400, Huntsville AL) and transfected into Swan71 cells according to the manufacturer's instructions. Swan71 cells were seeded in a 10-cm dish at a density of 5×10^6 cells. When the cells were 80% confluent, 10 µg of plasmid DNA (5:3:2, psPAX:FUGW:pMD2G) and 30 µL of polyethylenimine solution (1 mg/mL) were added. Virus was harvested at 48–72 h and concentrated by ultracentrifugation. Viral infection was performed in suspension using 10^6 cells with concentrated viral particles. The cells were incubated 1–2 h at 37 °C with intermittent shaking. After incubation, the cells were transferred to a 75-cm^2 tissue culture flask with 6 mL of fresh medium containing viral particles and placed in the incubator overnight. The next day, the cells were inspected under fluorescence microscopy, and *Axl* levels were analyzed by real-time (RT)-PCR and WB analysis to evaluate the infection efficiency. The viral medium was removed and replaced with fresh medium containing puromycin (1 µg/mL) to select infected cells.

Statistical analysis

Differences between means (three groups or more) were determined using analysis of variance, and differences between two groups were analyzed using either Student's *t*-test or a Mann–Whitney test, using GraphPad Prism 7 statistical software (La Jolla, CA). A p-value < 0.05 was considered statistically significant, and data are presented as the mean ± standard error of the mean or median with range.

RESULTS

LPS signaling induces type I IFNβ in trophoblast cells

Our first objective was to elucidate the LPS-TLR4 signaling pathway that was induced in trophoblast cells by exposing the first-trimester trophoblast cell line Swan71 to a low concentration of LPS (100 ng/mL), representing physiological levels of bacterial products released by commensal bacteria. In contrast to previous studies using higher concentrations of LPS (microgram concentrations),[32] TLR4 ligation by LPS at nanogram concentrations did not involve the induction of the classical MyD88/NF-κB-dependent pathway since we did not observe expression of NF-κB-related inflammatory cytokines; we even observed a decrease in the levels of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 (Supplementary fig. 1A). Instead, we detected a specific increase in IFN-related genes such as *CXCL10* (IP-10) and *RANTES* (Supplementary fig. 1B). Based on this cytokine profile, we hypothesized that in the trophoblast, LPS might induce type I IFN expression through the MyD88-independent pathway. Accordingly, we treated Swan71 trophoblast cells with LPS (100 ng/mL) and evaluated the mRNA expression of type I IFNα and IFNβ over time. As shown in Fig. 1a, LPS stimulation induced a time-dependent but transient increase in IFNβ, which peaked between 2 and 3 h and thereafter decreased (Fig. 1a). Interestingly, the effect was specific for IFNβ since we did not observe an induction of IFNα mRNA expression (Fig. 1b). We then tested whether the increase in mRNA levels translated into protein expression. Thus, we evaluated intracellular protein expression of IFNβ in trophoblasts over time by WB analysis, which increased following TLR4 ligation by LPS in a time-dependent manner (Fig. 1c, d). Furthermore, when we evaluated the supernatants of trophoblast cells exposed to LPS (100 ng/mL) collected at different time points by ELISA, we found that secreted IFNβ protein levels increased as early as 12 h and further accumulated at 48 and 72 h post LPS treatment, while no secreted IFNβ was detected in the control group (Fig. 1e). These results demonstrate that LPS is able to promote specific IFNβ expression at the mRNA, protein and secretory levels in trophoblast cells.

We then examined whether the in vitro findings could also be observed in vivo by evaluating IFNβ expression in the placenta of C57BL/6 mice exposed to low concentrations of LPS (20 µg/kg) at day ED15.5. Concentrations of LPS above 100 µg/kg are known to induce labor and fetal death, while the same LPS at concentrations of 20 µg/kg are not detrimental for pregnancy.[9] First, we determined *ifnβ* mRNA expression by qPCR and observed a 3.5-fold increase in the placenta of LPS-treated mice when compared to the basal levels observed in the control group as early as 10 h after treatment (Fig. 2a). At the protein level, we detected IFNβ expression in lysates obtained from placenta of control and LPS-treated mice by WB analysis (Fig. 2b). However, we did not observe a significant increase in the intracellular protein levels in response to LPS treatment, potentially due to the increased secretion triggered by LPS treatment, as described in the in vitro experiments.

These findings reveal a potential role for bacterial signals through TLR4 as providers of tonic stimulation for the expression of type I IFNβ in the placenta. We confirmed this hypothesis by comparing the basal expression levels of IFNβ mRNA levels in placental samples collected from untreated WT, *Tlr4*$^{-/-}$ (lack TLR4 signal) and *Trif*$^{-/-}$ (cannot activate TBK/TRIF and cannot induce the MyD88-independent pathway) mice at E17.5 (prior to delivery). We observed a significant inhibition of IFNβ mRNA expression in both *Tlr4*$^{-/-}$ and *Trif*$^{-/-}$ murine placenta compared with WT placenta (Fig. 2c) and no induction of IFNβ expression in *Tlr4*$^{-/-}$ and *Trif*$^{-/-}$ mice following treatment with LPS (data not shown). These data demonstrate, in vitro and in vivo, that the main response of the placenta to low concentrations of LPS is the production of type I IFNβ and requires TLR4 as well as a functional TRIF pathway (Fig. 2c).

The induction of IFNβ expression in trophoblast cells is mediated by the TLR4/TBK/IRF3 pathway

After observing that TLR4 responses to low concentrations of LPS in placenta were associated with IFNβ expression rather than classical MyD88/NFκB-inflammatory cytokines, we tested the hypothesis that in trophoblast cells, LPS may activate the MyD88-independent intracellular pathway, which involves the phosphorylation of 3-phosphoinositide-dependent kinase 1 (TBK), a kinase associated with the TRIF pathway, and IRF3, a major regulator of type I IFN expression.[33] We detected an increase in the phosphorylation of TBK (pTBK) and IRF3 (pIRF3) within 1 h of LPS exposure (Fig. 3a). To further confirm the involvement of TBK and IRF3 in TLR4-induced IFNβ expression in trophoblast cells, we exposed the cells to LPS (100 ng/mL) in the presence or absence of the pTBK inhibitor BX795.[34,35] BX795 is an aminopyrimidine compound that was developed as an inhibitor of the catalytic activity of TBK1 by blocking its phosphorylation.

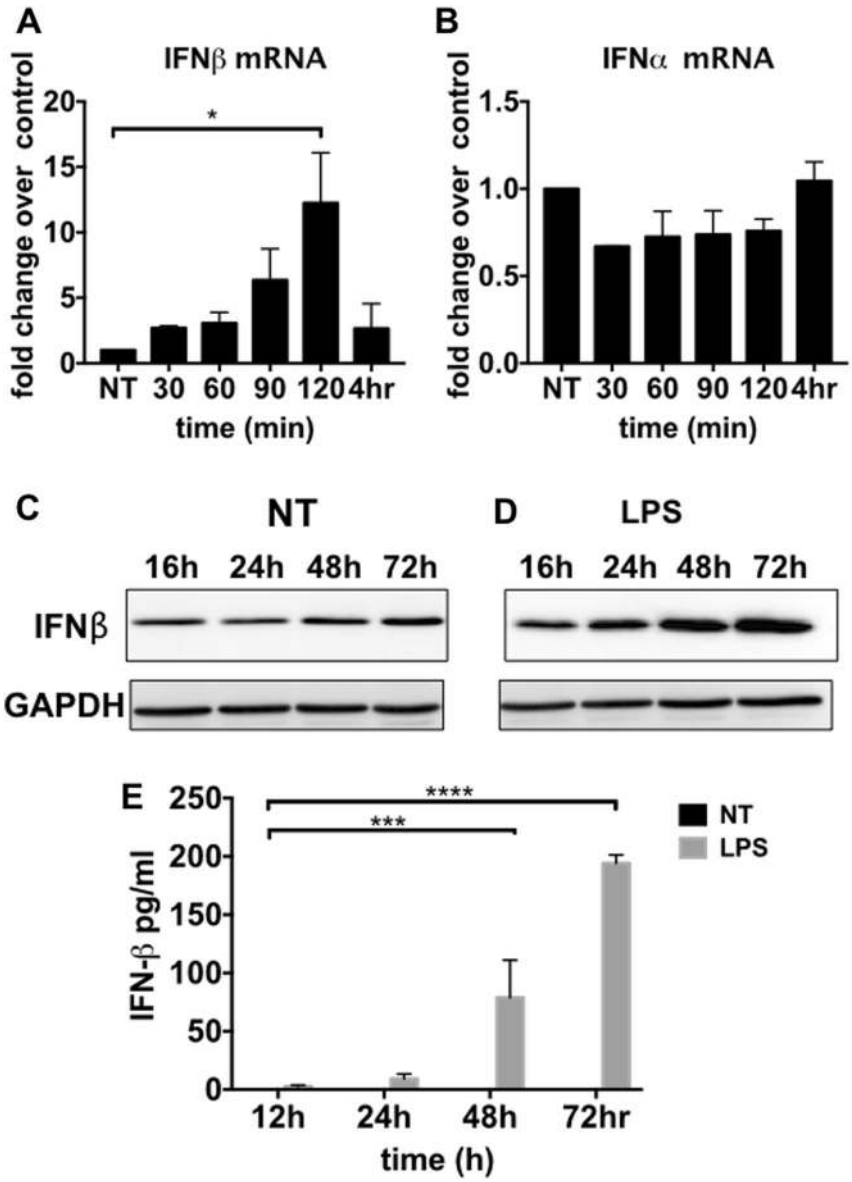


Fig. 1 Type I interferon beta is induced by LPS in trophoblast cells. **a**, **b** RNA analysis type I IFNβ expression. Swan71 cells were treated with LPS (100 ng/mL), and RNA was collected over time. Note that LPS induced a time-dependent increase in type I IFNβ but not in IFNα; *$p < 0.05$ by ANOVA; bar graph represents the mean ± s.e.m.; $n = 3$ independent experiments. **c**, **d** Western blot analysis of type I IFNβ intracellular expression in the first-trimester trophoblast cell line Swan71. IFNβ protein expression was evaluated in cells treated with LPS (100 ng/mL) over time. Note the presence of intracellular IFNβ expression (**c**) and the time-dependent increase in its expression following LPS treatment (**d**); representative experiment out of $n = 3$. **e** Evaluation of IFNβ secretion in the supernatants of trophoblast cells exposed to LPS. Cell-free supernatants were collected serially, and IFNβ levels were determined by ELISA. Despite intracellular protein expression, **c** trophoblast cells did not show constitutive IFNβ secretion (NT); however, upon LPS treatment, detectable levels of IFNβ were observed as early as 12 h and further accumulated at 48 and 72 h post LPS treatment. ***$p < 0.001$ and ****$p < 0.0001$ by ANOVA; bar graph represents the mean ± s.e.m., $n = 3$ independent experiments

Our results showed that inhibition of pTBK by BX795 prevented LPS-induced IFNβ expression (Fig. 3b), which indicated that the dominant pathway in trophoblast cells in response to a low concentration of LPS is the TLR4/TBK/IRF3/IFN pathway. These in vitro findings together with the in vivo data obtained from experiments using placental samples from *Trif*$^{-/-}$ mice (Fig. 2c) (Trif is the bridge between TLR4 and TBK1), showing no detectable basal levels of IFNβ, suggest that TLR4 signaling is responsible for maintaining basal IFNβ expression in the trophoblast.

IFNβ promotes the expression of ISGs in trophoblast cells

Having established that TLR4 ligation by LPS leads to rapid production of IFNβ, we next investigated the direct effect of IFNβ on trophoblast cells. IFNβ acts in both an autocrine and paracrine manner to induce the expression of ISGs that can then have immune regulatory functions.[2] Importantly, we have shown that the loss of IFNβ signaling in the placenta leads to uncontrolled viral replication and fetal viral infection;[27] therefore, understanding the factors that are induced by IFNβ in trophoblast is important to elucidate the mechanism associated with the antimicrobial responses induced by the placenta. Thus, we examined the gene expression profile of Swan71 trophoblast cells following exposure to IFNβ (125 IU) for 8 h using a human IFN immune response array specific for genes known to be regulated by type I IFNs (Type I Interferon Response PCR Array, Qiagen). As shown in Supplementary fig. 2A, IFNβ induced a wide range

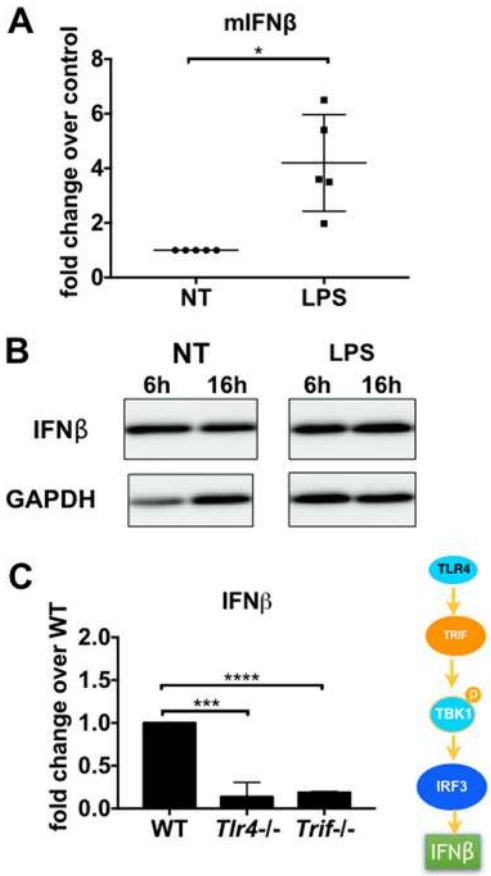


Fig. 2 Type I IFNβ expression in mouse placenta. **a** In vivo effect of low-dose LPS treatment in mouse placenta. C57BL/6 pregnant mice were injected with LPS (20 µg/kg) on day E15.5, and placental samples were collected 10 h later. Note the significant increase in IFNβ mRNA expression levels detected in placenta following LPS treatment. *$p < 0.05$ by *t*-test; $n = 5$/group. **b** Western blot analysis of IFNβ expression in mouse placental samples. IFNβ protein expression was evaluated in samples collected as described in (**a**), and protein expression was determined by western blot analysis. Representative experiment out of $n = 5$. **c** Evaluation of basal IFNβ expression in *Tlr4*$^{-/-}$ and *Trif*$^{-/-}$ mice. RNA was extracted from placenta obtained from WT, *Tlr4*$^{-/-}$, and *Trif*$^{-/-}$ mice at E17.5, and IFNβ expression was analyzed by qPCR. Note the absence of basal IFNβ expression in both KO mice compared with wild type. Data are representative of three pooled independent experiments. ***$p < 0.001$ and ****$p < 0.0001$ by the *t*-test compared with WT; $n = 3$ animals/group

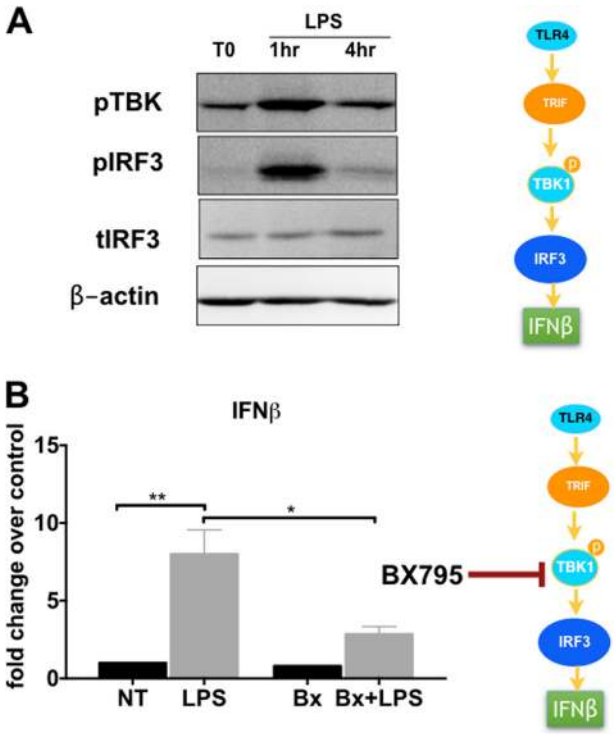


Fig. 3 LPS-TLR-4 ligation promotes IFNβ expression through the TBK-IRF3 pathway. **a** TLR4 ligation by LPS promotes TBK and IRF3 phosphorylation. Trophoblast Swan71 cells were treated with LPS (100 ng/mL) for 1 or 4 h, protein was extracted, and western blot analysis was performed to detect the phosphorylation of TBK and IRF3. Note the increase in pTBK and pIRF3 at 1 h after LPS treatment. Representative figure of five independent experiments. **b** Inhibition of pTBK blocks TLR4-induced IFNβ expression. Trophoblast Swan71 cells were pretreated with BX795, a pTBK inhibitor, for 2 h, followed by 100 ng/mL LPS for 4 h, after which RNA was extracted and IFNβ expression measured by qPCR. Note the lack of IFNβ expression following LPS treatment in the presence of BX795. *$p < 0.05$ and **$p < 0.01$ by ANOVA; bar graph represents the mean ± s.e.m.; $n = 3$ independent experiments

of genes associated with antiviral responses, innate immunity, the TLR pathway, inflammation (cytokines/chemokines), and antigen processing and presentation (Tables 2 and 3).

We next validated the findings from the array by testing the expression levels of representative ISGs with antimicrobial and cell survival roles, such as IRF7, ISG20, CXCL10, and TRAIL. Supplementary fig. 2B shows that IFNβ treatment (125 IU) of trophoblast cells stimulated a time-dependent induction of these ISGs (Supplementary fig. 2B-D); furthermore, IFNβ-induced mRNA expression was followed by protein expression of these ISG, as shown for IRF7 (WB) and CXCL10 (Luminex) (Supplementary fig. 3A).

We further validated the specificity of this effect by exposing trophoblast cells to increasing concentrations of IFNβ, and as shown in Supplementary fig. 3B-C, we observed a dose-dependent induction of CXCL10, ISG20, and TRAIL mRNA within 8 h (Supplementary fig. 3B-C). Specifically, these findings suggest that IFNβ, acting in a paracrine or autocrine manner, induces the expression of ISGs in trophoblast, which might function as a mediator of immune protection and immune regulation during pregnancy.

Negative regulation of IFNβ by TAM receptors in human trophoblasts

The IFN response is tightly regulated, and shortly after IFN exposure, cells enter a state known as IFN desensitization that can last up to several days. The physiological process of desensitization allows cells to recover from IFN signaling, whereas dysregulation of IFN production and signaling is manifested in immunologic disorders.[22] This is especially true in the placenta since an uncontrolled IFNβ response can be detrimental to pregnancy. We observed that IFNβ treatment (12.5 IU) of trophoblast cells induced the expression of TAM receptor Axl and SOCS1 protein (Supplementary fig. 4 and Table 2), two ISGs that can negatively regulate IFN signaling.[36]

SOCS1 has been characterized as a negative feedback loop for type I IFN signaling;[37] however, the induction of SOCS proteins by IFNAR activation has been shown to progress through and be contingent on TAM receptor activation.[24] To address whether TAM receptors play a role in the process of IFN desensitization and might be responsible for the negative feedback observed in LPS-induced IFNβ expression shown in Fig. 1a (i.e., a significant decrease at 4 h), we first assessed the expression of the TAM

Table 2. Human type 1 interferon array analysis performed on trophoblast cell treated with 125 IU IFNβ for 8 h

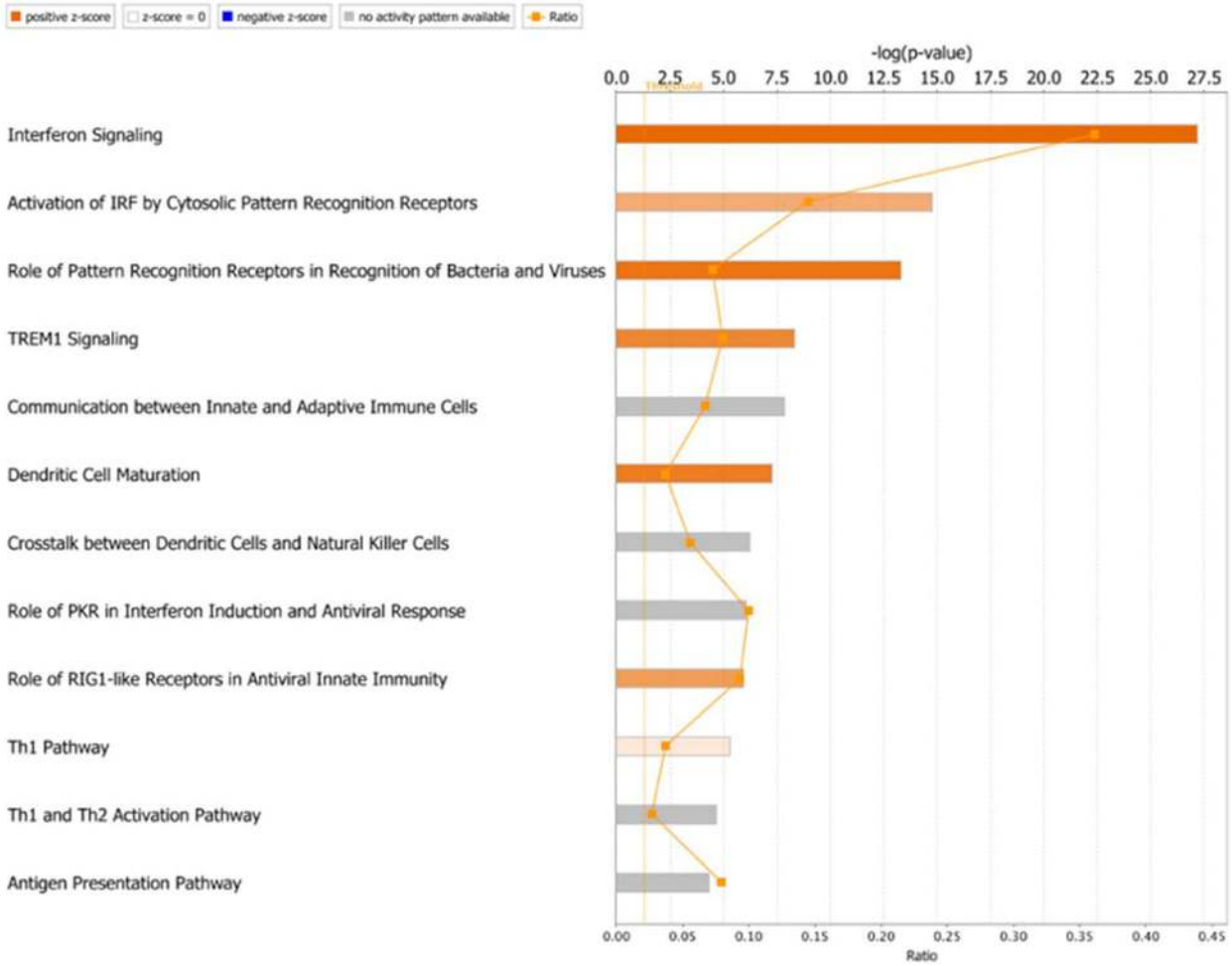
Gene	Gene description	Fold change
CXCL10	Chemokine (C-X-C motif) ligand 10	66.97
ISG20	Interferon stimulated exonuclease gene 20 kDa	25.87
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	20.23
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	19.03
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	8.94
OAS2	2′-5′-oligoadenylate synthetase 2, 69/71 kDa	8.44
IFIH1	Interferon induced with helicase C domain 1	8.16
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	8.15
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	8.06
OAS1	2′-5′-oligoadenylate synthetase 1, 40/46 kDa	7.50
TAP1	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	7.42
IFI30	Interferon, gamma-inducible protein 30	7.28
GBP1	Guanylate binding protein 1, interferon-inducible	7.24
ISG15	ISG15 ubiquitin-like modifier	7.20
MX2	Myxovirus (influenza virus) resistance 2 (mouse)	6.86
TLR3	Toll-like receptor 3	6.31
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	6.04
BST2	Bone marrow stromal cell antigen 2	5.87
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	5.26
CIITA	Class II, major histocompatibility complex, transactivator	4.89
MYD88	Myeloid differentiation primary response gene (88)	4.82
STAT1	Signal transducer and activator of transcription 1, 91 kDa	4.58
IFI16	Interferon, gamma-inducible protein 16	4.16
STAT2	Signal transducer and activator of transcription 2, 113 kDa	4.08
IFITM1	Interferon induced transmembrane protein 1 (9–27)	3.86
NMI	N-myc (and STAT) interactor	3.71
PML	Promyelocytic leukemia	3.61
SOCS1	Suppressor of cytokine signaling 1	3.55
CCL2	Chemokine (C-C motif) ligand 2	3.46
IRF7	Interferon regulatory factor 7	3.32
JAK2	Janus kinase 2	3.25
TMEM173	Transmembrane protein 173	3.22
IRF1	Interferon regulatory factor 1	3.19
HLA-E	Major histocompatibility complex, class I, E	2.85
IFNB1	Interferon, beta 1, fibroblast	2.76
IFI6	Interferon, alpha-inducible protein 6	2.69
CCL5	Chemokine (C-C motif) ligand 5	2.33
ADAR	Adenosine deaminase, RNA-specific	2.29
HLA-A	Major histocompatibility complex, class I, A	2.28
IFI27	Interferon, alpha-inducible protein 27	2.07

Listed genes are those that were twofold or higher

receptors Axl, Mer, and Tyro in trophoblast cells isolated from first-trimester placentas (cytotrophoblast and syncytiotrophoblast) by western blotting and found that Axl and Mer, but not Tyro, were expressed (Supplementary fig. 5). These findings were confirmed in human first-trimester placental samples immuno-stained for Axl and Mer. First-trimester placental villi were positive for both Axl and Mer, showing strong staining in the cytotrophoblast and syncytiotrophoblast (Fig. 4a and Supplementary fig. 6). Interestingly, Mer expression was observed in the decidua. (Fig. 4a, arrows).

We further validated the immunofluorescence findings by evaluating Axl and Mer mRNA and protein expression in the first-trimester human trophoblast cell line Swan71 by qPCR and WB analysis. Our data showed that similar to the first-trimester trophoblast primary cultures, Swan71 trophoblast cells expressed Axl and Mer mRNA. Interestingly, Axl mRNA levels were much higher than Mer mRNA levels when compared to in vitro-differentiated macrophages (Fig. 4b, c). Finally, Swan71 trophoblast cells also expressed high mRNA levels of Gas6, the ligand for Axl (Fig. 4d). The mRNA expression was confirmed at the protein level by ELISA, for which we observed constitutive secretion of Gas6 protein by trophoblast cells (Fig. 4e). The above data demonstrate that trophoblast cells express the TAM receptors Axl and Mer, as well as the ligand Gas6.

Table 3. Ingenuity pathway analysis indicating pathways enriched in trophoblast cells exposed to type 1 interferon beta. Ratio indicates number of twofold differentially expressed genes that map to a pathway divided by the total number of genes in human interferon array



Next, we assessed the functionality of the TAM receptors by incubating Swan71 cells with human recombinant Gas6 (ligand for Axl) and determining the expression of SOCS1, a known downstream target of the TAM receptor pathway and inhibitor of IFNβ signaling. As shown in Supplementary fig. 7, Gas6 treatment of trophoblast induced a significant increase in SOCS1 mRNA after 6 and 24 h of treatment, confirming that TAM receptors were functional and could play a regulatory role in IFNβ responses in trophoblast cells.

The induction of fetal demise in *AxlMer*$^{-/-}$ pregnnt mice is associated with higher IFNβ expression levels

To determine the potential role of TAM receptors in regulating IFNβ expression through the TLR4/IFNβ pathway during pregnancy, we used double TAM receptor knockout mice lacking Axl and Mer (*AxlMer*$^{-/-}$), the two receptors found to be expressed in trophoblast. We exposed WT and *AxlMer*$^{-/-}$ pregnant mice to a low concentration of LPS (20 µg/kg) at ED15.5 by i.p. injection, which as shown in Fig. 2a induced IFNβ expression and had no effect on pregnancy outcome (Fig. 5a). Interestingly, LPS (20 µg/kg) induced fetal demise in approximately 60% of *AxlMer*$^{-/-}$ mice at 48 h post LPS treatment, while the same dose had no effect on WT mice (Fig. 5b, c). Histopathologic examination of the placenta obtained from *AxlMer*$^{-/-}$ pregnant mice sacrificed 21 h after administration of LPS (20 µg/kg) revealed major morphological changes, including hemorrhage, swelling in the spongiotrophoblast layer, and disruption of the trophoblast giant cell layer (Fig. 5d, e). These morphological changes are suggestive of a process of cell death within the placenta that could explain the observed fetal demise.

To determine whether an inflammatory process was responsible for the observed morphological changes, we evaluated the expression of two major mediators of inflammation, IL-1β and TNFα, in placentas obtained from WT and *AxlMer*$^{-/-}$ pregnant mice. As expected, we did not observe an induction of IL-1β and TNFα in the placentas of WT mice at a concentration of 20 µg/kg; however and surprisingly, we also did not detect a pro-inflammatory response in placentas from *AxlMer*$^{-/-}$ pregnant mice following LPS treatment at the same concentration (Supplementary fig 8A-B), suggesting that the detrimental effect on the pregnancy observed in *AxlMer*$^{-/-}$ pregnant mice was not due to a pro-inflammatory storm.

Since it has been shown that high levels of IFNβ expression can induce cell death or apoptosis,[38,39] we evaluated whether the lack of Axl and Mer receptors was associated with increased expression of IFNβ in placenta. Indeed, as shown in

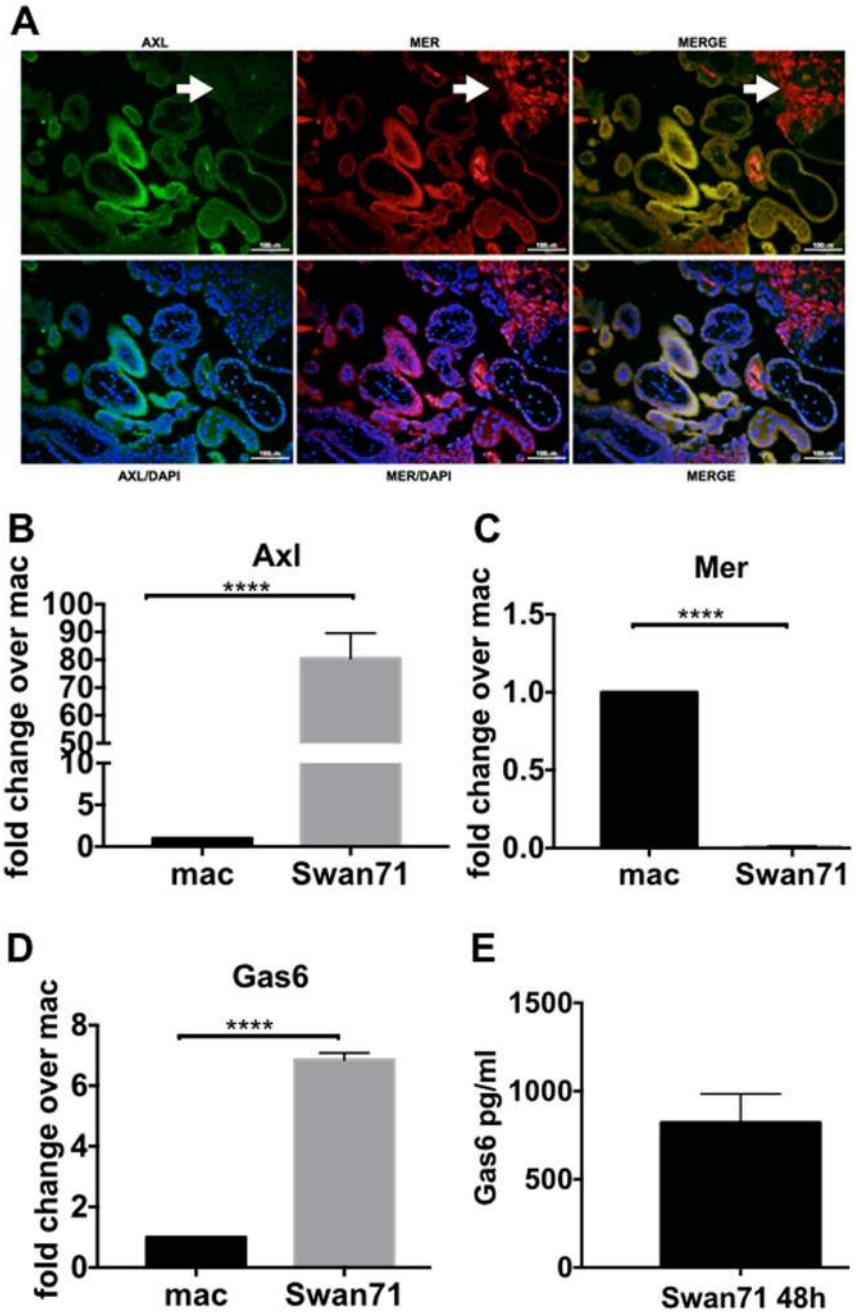


Fig. 4 TAM receptors and their ligand Gas6 are present and functional in human placenta. **a** Immunohistochemical analysis of first-trimester placentas for Axl and Mer expression. Tissues were stained with specific antibodies for Axl (top/bottom left panel), Mer (top/bottom middle panel), and DAPI to indicate cell nuclei (blue). Merged images (top and bottom right panels) demonstrated positive staining for membranous expression of Axl and Mer receptors in cytotrophoblasts and syncytiotrophoblast. Interestingly, decidua were mainly positive for Mer antibody (arrow). Representative figure of 10 samples. **b**–**d** Relative mRNA expression levels of Axl Mer and Gas6 in trophoblast cells. mRNA was extracted from untreated trophoblast Swan71 cells and in vitro-differentiated macrophages (positive control), and qPCR was performed to measure the expression of Axl (**b**), Mer (**c**), and Gas6 (**d**). Results are presented relative to those of macrophages, set as 1. ****$p < 0.0001$ by the *t*-test; the bar graph represents the mean ± s.e.m.; $n = 3$ independent experiments. **e** Expression levels of secreted Gas6 by trophoblast cells. Cell-free medium from untreated trophoblast Swan71 cultured for 48 h was collected and analyzed for levels of GAS6 using the Simple Plex Assay. Gas6 is constitutively secreted from trophoblasts cells. The bar graph represents the mean ± s.e.m.; $n = 3$ independent experiments

Fig. 6a, placentas obtained from untreated *AxlMer*$^{-/-}$ pregnant mice showed significantly higher levels of basal *ifnβ* expression (Fig. 6a) and downstream ISGs such as ISG20 (Supplementary fig. 8C), compared to untreated WT mice. Moreover, TLR4 activation with LPS (20 µg/kg) in *AxlMer*$^{-/-}$ pregnant mice not only further increased IFNβ expression levels in placenta (Fig. 6b) but also induced the expression of ISGs associated with the regulation of apoptosis. Thus, compared with WT animals, we observed a significant increase in *trail* and *fas* mRNA expression in placenta of the *AxlMer*$^{-/-}$ group following TLR4 activation with LPS (20 µg/kg) (Fig. 6c, d). Furthermore, we observed a positive correlation between high levels of *ifnβ* expression and increased expression of the IFNβ-induced gene *trail* (Fig. 6e).

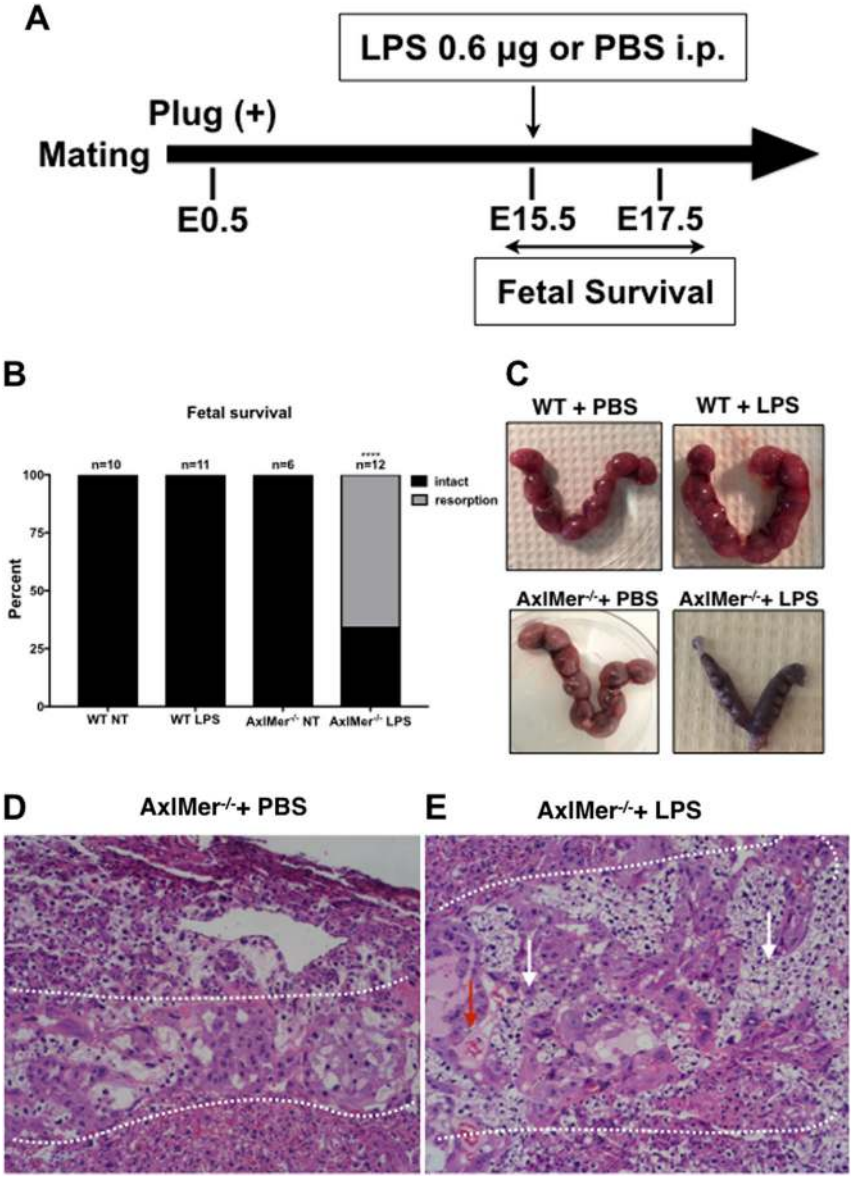


Fig. 5 Fetal demise is induced in *AxlMer*$^{-/-}$ pregnant mice in response to low-dose LPS. **a** Experimental design for in vivo evaluation of the effect of LPS on pregnancy. Pregnant mice were administered LPS (20 µg/kg) intraperitoneally (i.p.) at E15.5, and then fetal outcome was observed for 48 h. **b** Low-dose LPS induces fetal demise in *AxlMer*$^{-/-}$. Pregnant *AxlMer*$^{-/-}$ mice, but not WT mice, exhibit poor fetal outcome following treatment with low doses of LPS. ****$p < 0.0001$ by the chi-square test. WT wild type, PBS phosphate-buffered saline, LPS lipopolysaccharide. **c** Morphological evaluation of the placenta and decidua from pregnant *AxlMer*$^{-/-}$ and WT mice. Gestational sacs from pregnant mice were removed and the morphology evaluated: WT mice treated with PBS or LPS appeared normal (top left and right) similar to *AxlMer*$^{-/-}$ pregnant mouse treated with PBS (C; bottom left), whereas it was empty in *AxlMer*$^{-/-}$ pregnant mice treated with low-dose LPS (**c**; bottom right). Representative experiment out of $n = 6$ or 12 as stated in (**b**). **d**, **e** Histological changes in placenta from WT and pregnant *AxlMer*$^{-/-}$ mice. WT and pregnant *AxlMer*$^{-/-}$ mice were administered PBS or LPS (20 µg/kg) i.p. at E15.5, and the animals were sacrificed 21 h post treatment. Histological examination of the placenta delineated major morphological changes, including hemorrhage, swelling in the spongiotrophoblast layer (**e**, white broken lines) and disruption of the trophoblast giant cell layer (**e**, white arrows). Representative image out of $n = 6$ or 12 as stated in (**b**)

To determine whether there was a correlation between the observed increase in TRAIL and FAS and the presence of placental apoptosis, we quantified the activity of caspase-8 (extrinsic pathway induced by FAS and TRAIL)[40] in placental samples collected from WT and *AxlMer*$^{-/-}$ pregnant mice at 21 h post TLR4 activation with LPS (20 µg/kg). Our data showed that concomitant with the significant increase in *fas* expression, caspase-8 activation was also significantly increased in *AxlMer*$^{-/-}$ pregnant mice (Fig. 6f). Taken together, these findings demonstrate that in the absence of Axl/Mer regulatory signals, there is a dysregulation of IFNβ expression characterized by increased baseline production and an exacerbated IFNβ response to TLR4 activation with LPS. The outcome of this intensified IFNβ production is trophoblast apoptosis and fetal demise.

In vitro inhibition of Axl receptor expression in trophoblast cells results in dysregulated IFNβ production

To further substantiate the role of TAM receptors in the regulation of IFNβ expression in trophoblast cells and elucidate the specific role of Axl and Mer, we inhibited the expression of either Axl or Mer in Swan71 cells using specific shRNAs. We observed that the absence of Axl was sufficient to affect IFNβ expression. Indeed, in

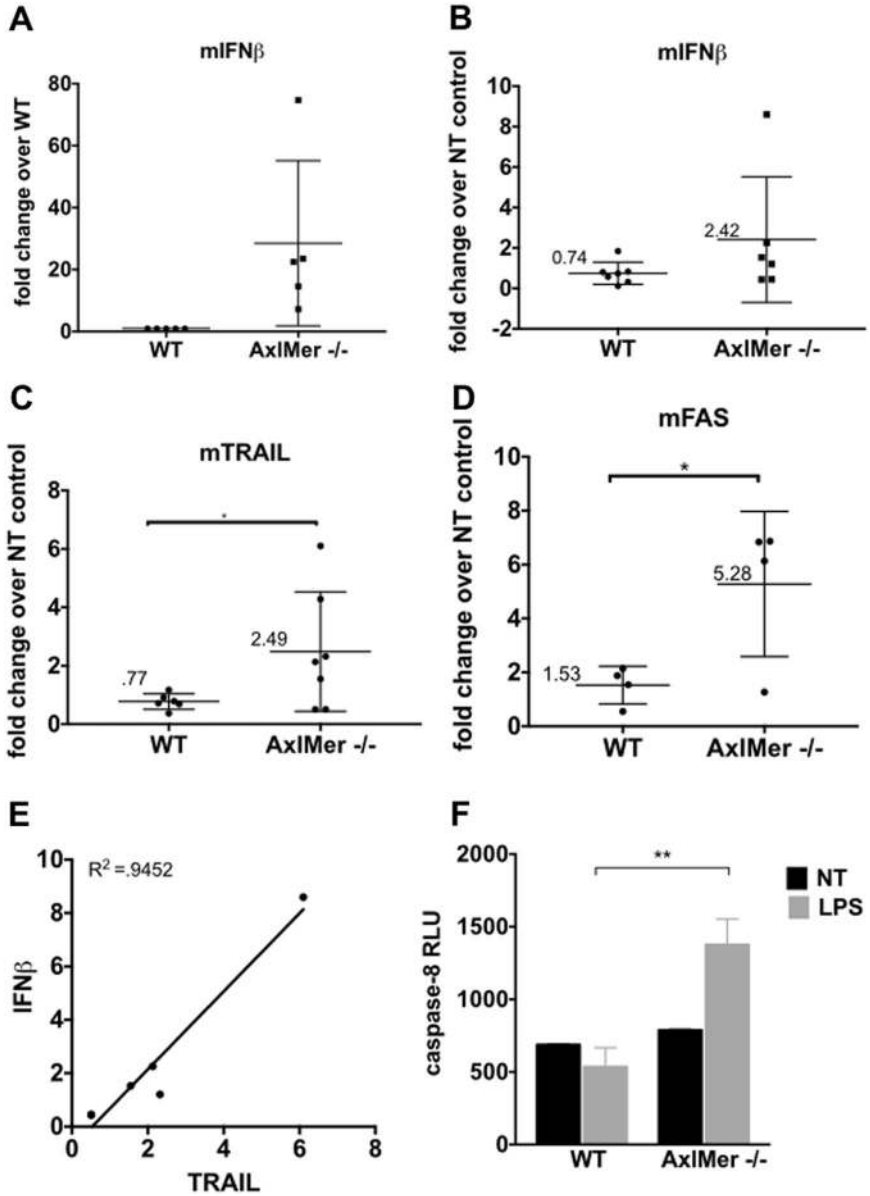


Fig. 6 Absence of TAM receptor in trophoblast is associated with dysregulated IFNβ production and of ISGs in placenta. **a** Enhanced IFNβ expression in the placenta of *AxlMer*$^{-/-}$ mice. Samples of placenta obtained from E15.5 *AxlMer*$^{-/-}$ mice exhibited higher IFNβ mRNA expression levels compared with the WT control; $n = 5$ animals/group. **b**–**d** Dysregulation of IFNβ expression and increased expression of pro-apoptotic ISGs in *AxlMer*$^{-/-}$ mice in response to LPS. WT or *AxlMer*$^{-/-}$ mice were treated i.p. with PBS or LPS (20 μg/kg) on E15.5 and sacrificed after 6 h. Note the increased *ifnβ* (**b**) and higher fold change in pro-apoptotic *trail* (**c**) and *fas* (**d**) mRNA expression in *AxlMer*$^{-/-}$ placentae following LPS treatment as compared to wild-type mice; *$p < 0.05$ by the Mann–Whitney test; $n = 4$–6 mice/group. **e** Correlation between IFNβ and TRAIL expression levels. Positive correlation between IFNβ expression and the pro-apoptotic protein TRAIL in placental samples collected from *AxlMer*$^{-/-}$ mice following LPS treatment as described in (**b**); calculated by Pearson's correlation; $r = 0.972$; $p < 0.05$. **f** Increased caspase-8 activity in *AxlMer*$^{-/-}$ mice exposed to LPS. WT or *AxlMer*$^{-/-}$ mice were treated i.p. with PBS or LPS (20 μg/kg) on E15.5 and sacrificed after 21 h. Note the increase in caspase-8 in *AxlMer*$^{-/-}$ placentae following LPS treatment compared to wild-type mice; **$p < 0.01$ by ANOVA; the bar graph represents the mean ± s.e.m.; $n = 6$ mice/group

trophoblast cells lacking Axl, the baseline IFNβ expression was significantly higher than in the control cells (Fig. 7a). Therefore, in the following experiments, we focused on the role of Axl in the regulation of IFNβ expression in trophoblast cells. We established a trophoblast cell line lacking Axl by stable transfection of the Axl shRNA green fluorescent protein (GFP) vector into Swan71 cells (Swan71-shAxl). The establishment of a pure culture of Swan71-shAxl (lacking Axl) was validated by GFP expression, confirming that >90% of the cells were GFP-positive (Supplementary fig. 9A) and lacked Axl protein and RNA expression (Supplementary fig. 9B-C).

Next, we evaluated whether the lack of Axl and its effect on IFNβ expression in trophoblast cells might affect IFNβ expression following TLR4 stimulation with LPS between WT Swan71 (shCTL) and Swan71-shAxl. Our data showed that in addition to the augmented baseline IFNβ mRNA expression in Swan71-shAxl compared with WT cells (Fig. 7a), there was an enhancement of IFNβ mRNA expression following TLR stimulation with LPS (Fig. 7b). Moreover, Swan71-shAxl trophoblast secreted fivefold higher levels of IFNβ compared with shCTL trophoblast after TLR4 stimulation with a low dose of LPS (100 ng/mL) (Fig. 7c). In summary, these findings demonstrate that Axl is critical for

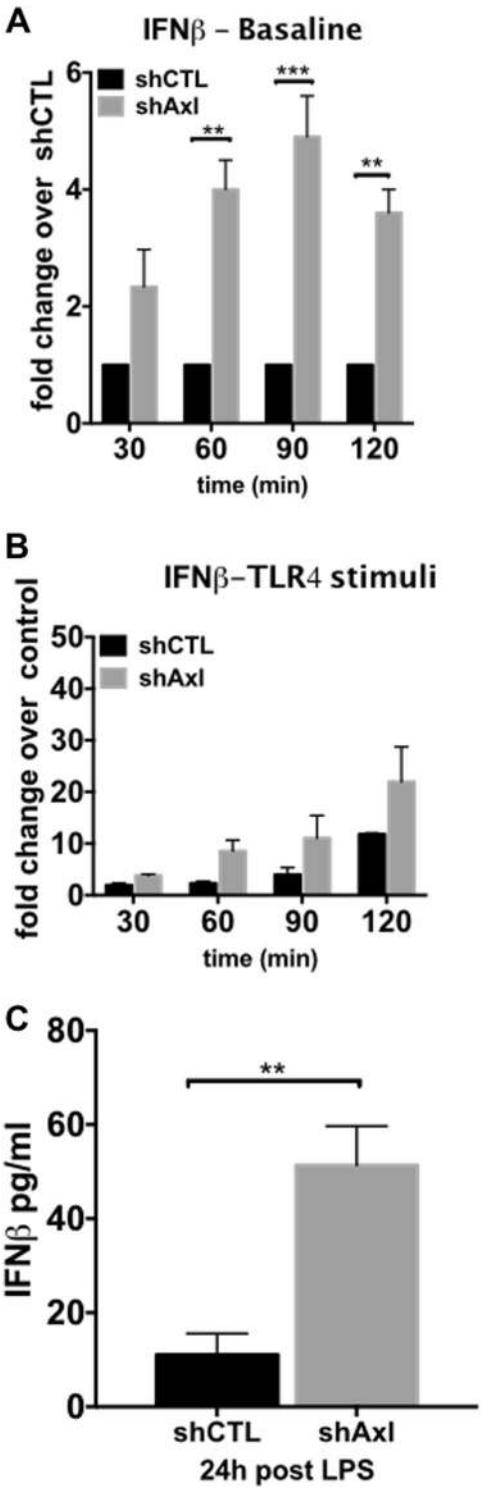


Fig. 7 Knockdown of Axl receptor expression in Swan71 cells by lentivirus infection results in dysregulation of IFNβ production. **a** Dysregulated expression of IFNβ in trophoblast cells lacking Axl. Trophoblast Swan71 cells were transfected with shRNA-Axl or vector control followed by determination of IFNβ mRNA levels after different times in culture. Note the higher IFNβ mRNA expression levels in trophoblast cells transfected with shRNA-Axl compared with the control; $^{**}p < 0.01$ and $^{***}p < 0.001$ by two-way ANOVA; the bar graph represents the mean ± s.e.m.; $n = 3$ independent experiments. **b** Lack of negative feedback on IFNβ expression in response to LPS in cells lacking Axl. Trophoblast Swan71 cells transfected with shRNA-Axl or vector control (shCTL) were treated with LPS (100 ng/mL) for different time periods followed by the determination of IFNβ expression by qPCR. Both cell types showed increases in IFNβ after approximately 90–120 min; however, in contrast to the control, LPS induced a significant and maintained increased IFNβ expression even after 120 min; $n = 3$ independent experiments. **c** Increased IFNβ secretion in trophoblast cells lacking Axl. shCTL and shAxl-Swan71 cells were treated with LPS (100 ng/mL), and IFNβ secretion was determined by ELISA. LPS treatment of shAxl-Swan71 was characterized by augmented IFNβ secretion in comparison to the control. $^{**}p < 0.01$ by two-way ANOVA; the bar graph represents the mean ± s.e.m.; $n = 3$ independent experiments

regulating IFNβ expression, and the absence of Axl is associated with augmented IFNβ production, both basal and in response to a TLR4 stimulus.

Inhibition of Axl receptor expression in trophoblast cells results in IFNβ-induced apoptosis

We previously reported that high levels of IFNβ induce apoptosis in trophoblast cells;[38] therefore, we hypothesized that the observed dysregulation of IFNβ expression in *AxlMer*$^{-/-}$ pregnant mice might be responsible for the presence of apoptosis and apoptosis-related genes in placentas obtained from *AxlMer*$^{-/-}$ pregnant mice. To test this hypothesis, we used the in vitro model of trophoblast cells lacking Axl and assessed whether the induction of IFNβ expression by stimulation of TLR4 with LPS could affect trophoblast survival. As shown in Fig. 8, live imaging analysis revealed that induction of IFNβ expression in the absence of Axl in Swan71-shAxl was associated with significant inhibition of cell growth (Fig. 8a, b) and a time-dependent increase in pro-apoptotic TRAIL mRNA expression levels (Fig. 8c). These in vitro findings correlated with the observations described in the animal studies.

To further confirm that the increase in apoptosis observed in Swan71-shAxl was due to the dysregulation of IFNβ expression, we treated WT Swan71 with high doses of IFNβ (1500 IU) and monitored cell viability and caspase activation. Our findings demonstrated that high doses of IFNβ induced trophoblast apoptosis through the activation of caspase 3 (Supplementary fig. 10A-C). These results conclusively demonstrated the importance of Axl receptor signaling in regulating IFNβ expression in trophoblasts.

High levels of IFNβ induces fetal demise

Finally, we tested whether high levels of IFNβ expression and subsequent activation of pro-apoptotic ISGs, as observed in *AxlMer*$^{-/-}$ pregnant mice, were responsible for fetal demise in vivo. Poly(I:C) is a double-stranded RNA viral mimic that is capable of eliciting strong type I IFN responses.[41] The main effect of poly(I:C) is to enhance IFNβ expression followed by IFNAR-induced ISG expression. To elucidate the effect of IFNβ, we selected two concentrations of poly(I:C), a low concentration (2.5 mg/kg), which has a minimal effect on the induction of IFNβ expression and secretion, and a high concentration (15 mg/kg), which induces robust IFNβ expression and downstream ISGs. Thus, pregnant mice were treated with poly(I:C) via intraperitoneal injection; 24 h later, the animals were sacrificed, and fetal development was evaluated. As shown in Fig. 9, the animals that received the high dose of poly(I:C) (15 mg/kg) showed generalized fetal death, resorption, and abnormal fetal development (Fig. 9a), which was not observed for the animals that received the low concentration of poly(I:C) (2.5 mg/kg). We confirmed the effect of poly(I:C) on IFNβ expression by determining the expression levels of IFNβ in placental samples obtained from mice exposed to the two concentrations of poly(I:C). As expected, we observed a robust IFNβ response in all placental samples exposed to poly(I:C) at the

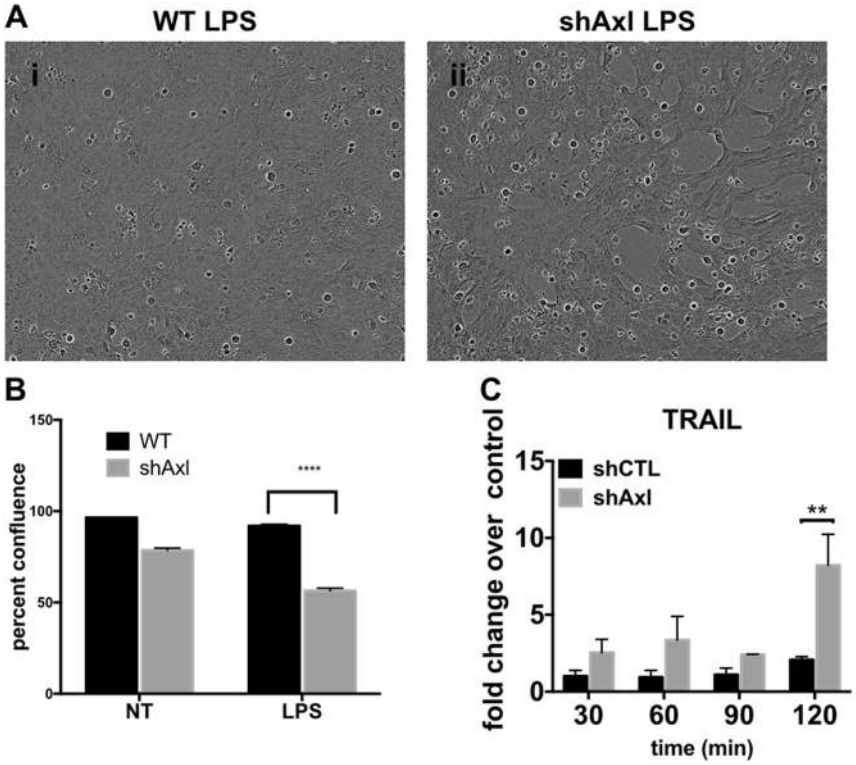


Fig. 8 The absence of Axl expression is associated with apoptosis of trophoblast cells in response to LPS. **a** Morphological changes in trophoblast cells in response to LPS. Trophoblast Swan71 cells transfected with shRNA-Axl or vector control were treated with LPS (100 ng/mL) and their growth monitored using cell imaging. Note the presence of apoptotic cells in the shRNA-Axl cell cultures, but not in the control, after LPS treatment; representative image out of $n = 3$. **b** Quantification of cell confluence by IncuCyte live cell imaging. Swan71 cells transfected with shRNA-Axl showed a significant decrease in cell confluence following treatment with LPS. ****$p < 0.0001$ by two-way ANOVA; the bar graph represents the mean ± s.e.m.; $n = 3$ independent experiments. **c** LPS treatment of shRNA-Axl trophoblast cells is associated with an increase in TRAIL expression. Swan71 cells transfected with shRNA-Axl showed increased levels of TRAIL mRNA expression in a time-dependent manner following LPS treatment; **$p < 0.01$ by two-way ANOVA; the bar graph represents the mean ± s.e.m.; $n = 3$ independent experiments

high but not at the lower poly(I:C) concentration (Fig. 9b). Furthermore, *trail* expression levels were significantly elevated in placentas from animals that received the higher concentration of poly(I:C) (Fig. 9c). These data demonstrate that signals that induce high levels of IFNβ expression and its downstream ISGs are detrimental for pregnancy, further supporting the role of IFNβ regulatory pathways in the protection of fetal survival.

DISCUSSION

The present study describes, for the first time, mechanisms that regulate IFNβ expression and function in the placenta. We demonstrate that (i) TLR4 activation in trophoblast cells induces the expression of type I IFNβ and is mediated by the MyD88-independent TRIF/TBK/IRF3 signaling pathway; (ii) IFNβ signaling in trophoblast promotes downstream ISG expression as well as negative feedback regulatory proteins such as the TAM receptor Axl; (iii) the TAM receptors Axl and Mer are expressed in placenta and are important modulators of IFN function by regulating IFNβ expression and ISGs; and (iv) the absence of Axl in vitro and in vivo is associated with an increased IFNβ baseline level and in the response to TLR4 stimulation. Furthermore, the augmented IFNβ expression in the absence of Axl and Mer in vivo is associated with fetal demise. Our findings describe potential mechanisms controlling IFNβ expression and function in placenta and the relevance of these positive and negative feedback effects to the success of pregnancy.

The placenta and specifically the trophoblast are important modulators of the immune response at the maternal–fetal interface.[42] Trophoblasts express TLR4 receptors that are able to produce cytokines and chemokines following activation.[43] However, it is interesting to note that trophoblasts are tolerant to bacterial products, as only high levels of LPS are able to trigger an inflammatory response,[9] suggesting the presence of a mechanism that tightly controls pro-inflammatory cytokine/chemokine production. A novel finding of this study is the demonstration that in trophoblasts, TLR4 activation by bacterial products such as LPS can promote IFNβ expression via the TLR4/TBK/IRF3 pathway. Although previous studies have demonstrated the ability of TLR4 to respond to LPS by inducing IFNβ expression through the TBK/IRF3 pathway,[33,44] this is the first study to show similar mechanisms in a developmental tissue, the placenta.

A growing body of evidence suggests that under homeostatic conditions the commensal microbiota, through TLRs, might be responsible for basal IFNβ production.[21,45] This is in agreement with our findings that ligation of TLR4 by LPS (at low concentrations) in trophoblast leads to activation of the TRIF-TBK1-IRF3 pathway; in the absence of TLR4 signaling (*Tlr4*$^{-/-}$) or TRIF (*Trif*$^{-/-}$), IFNβ expression is significantly diminished. Basal IFNβ expression has been shown to occur in healthy animals and, more importantly, has been demonstrated to be critical in immune cell function and responses to infection.[46] In immune cells, basal expression of IFNβ and the attendant tonic IFNAR signaling aids in the rapid mobilization of an antimicrobial response.[13] This basal IFNβ can provide the necessary protection against acute viral infections, and given that IFNβ is a known immune modulator,[13] basal IFNβ production can balance the

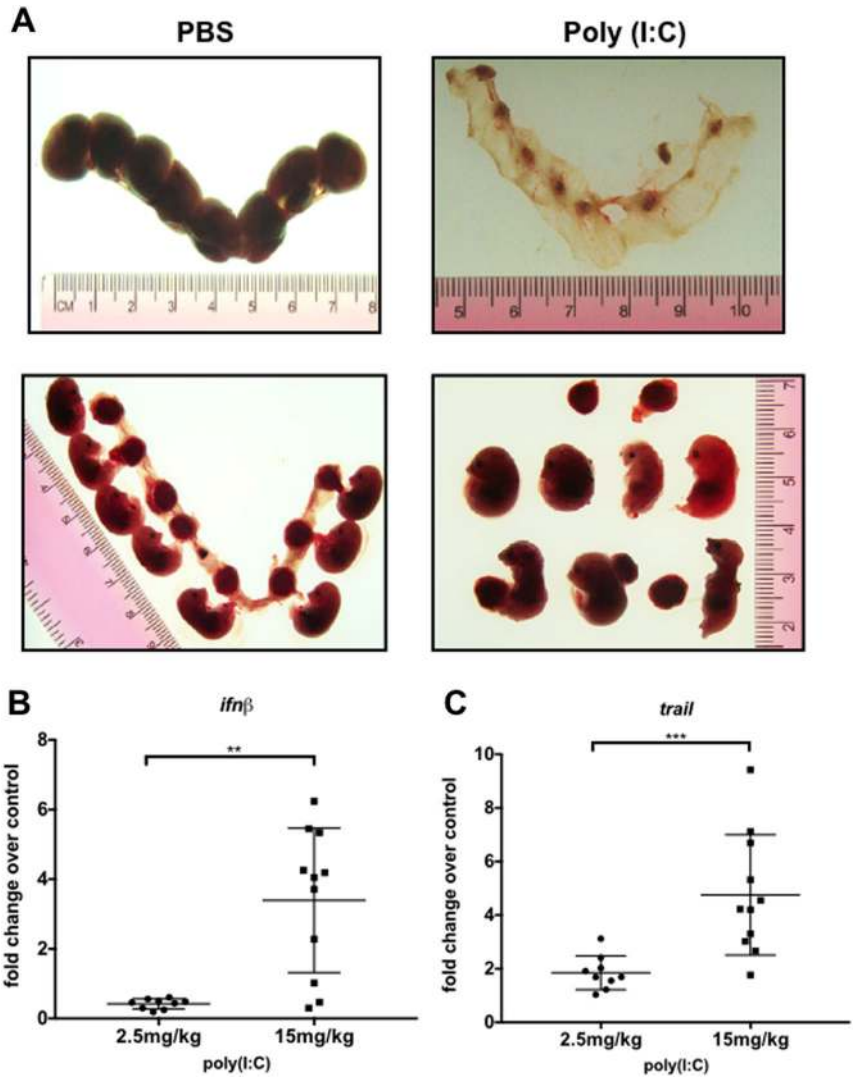


Fig. 9 Poly(I:C)-induced IFNβ is associated with fetal demise. **a** A high dose of poly(I:C) induces fetal demise in WT mice. Pregnant WT mice were treated with either PBS (Control) or poly(I:C) (15.5 mg/kg) i.p., and pregnancy outcome monitored for 24 h. Note the fetal demise in mice exposed to a high dose of poly(I:C). A low dose of poly(I:C) (2.5 mg/kg) resulted in a similar phenotype as the PBS control; representative image out of $n = 4$. **b**, **c** Increased IFNβ expression in the placenta of mice treated with a high dose of poly(I:C). WT mice were treated with PBS or with either 2.5 or 15.5 mg/kg poly(I:C) i.p on E15.5 and sacrificed after 4 h. Note the increased *ifnβ* (**b**) and higher fold change in pro-apoptotic *trail* (**c**) mRNA expression levels in WT placenta following poly(I:C) treatment at a high concentration (15 mg/kg) compared with 2.5 mg/kg; $^{**}p < 0.01$ and $^{***}p < 0.001$ by the Mann–Whitney test ($n = 9$–11/group)

immune response (tolerance to paternal antigens and protection against infections) at the maternal fetal interface.

During pregnancy, the importance of basal IFNβ and IFN signaling in the control of infection has been demonstrated in our previous studies, in which we showed that IFNAR$^{-/-}$ mice respond poorly to viral infection, and basal IFN signaling is downregulated due to viral infection-triggered inflammation in response to low doses of LPS.[27] Therefore, we can postulate that altering normal TLR4 responses in the placenta may cause a homeostatic imbalance of basal IFNβ expression, which in turn leads to aberrant immune responses to both viral and bacterial infections.

The expression of IFNβ in response to different stimuli needs to be strictly regulated to prevent potential toxic effects associated with chronic stimulation.[22] Shortly after IFNβ exposure, cultured cells enter what has been defined as an IFN-desensitized state that allows cells to recover from IFN signaling. Chronic expression of high levels of IFNβ may contribute to immune suppression by inhibiting the expression of cytokines and chemokines, but it can also be harmful by inducing apoptosis,[16] which is especially true in placenta since an uncontrolled virus-induced response can be detrimental to pregnancy.

Our data demonstrate a central role for TAM receptors, specifically Axl, during pregnancy as negative feedback loop regulators of IFNβ expression; its absence, furthermore, is associated with an augmentation of IFNβ/IFNAR signaling. The function of Axl as a regulator of IFNβ in the trophoblast described herein has important implications for our understanding of the differential responses to viral and bacterial infections during pregnancy.

TAM receptors are membrane tyrosine kinase receptors found in high abundance in immune cells and have been reported to regulate innate immune responses by dampening TLR signaling. Loss of TAM receptor signaling has been associated with stages of hypersensitive inflammatory responses implicated in sepsis, chronic inflammatory disease, and autoimmune diseases.[47,48] TAM receptor signaling has been found to inhibit TLR-induced inflammatory cytokines and, most notably, type I IFN receptor activation.[25,26] In this study, we characterized the expression of TAM receptors in trophoblast cells as well as in human

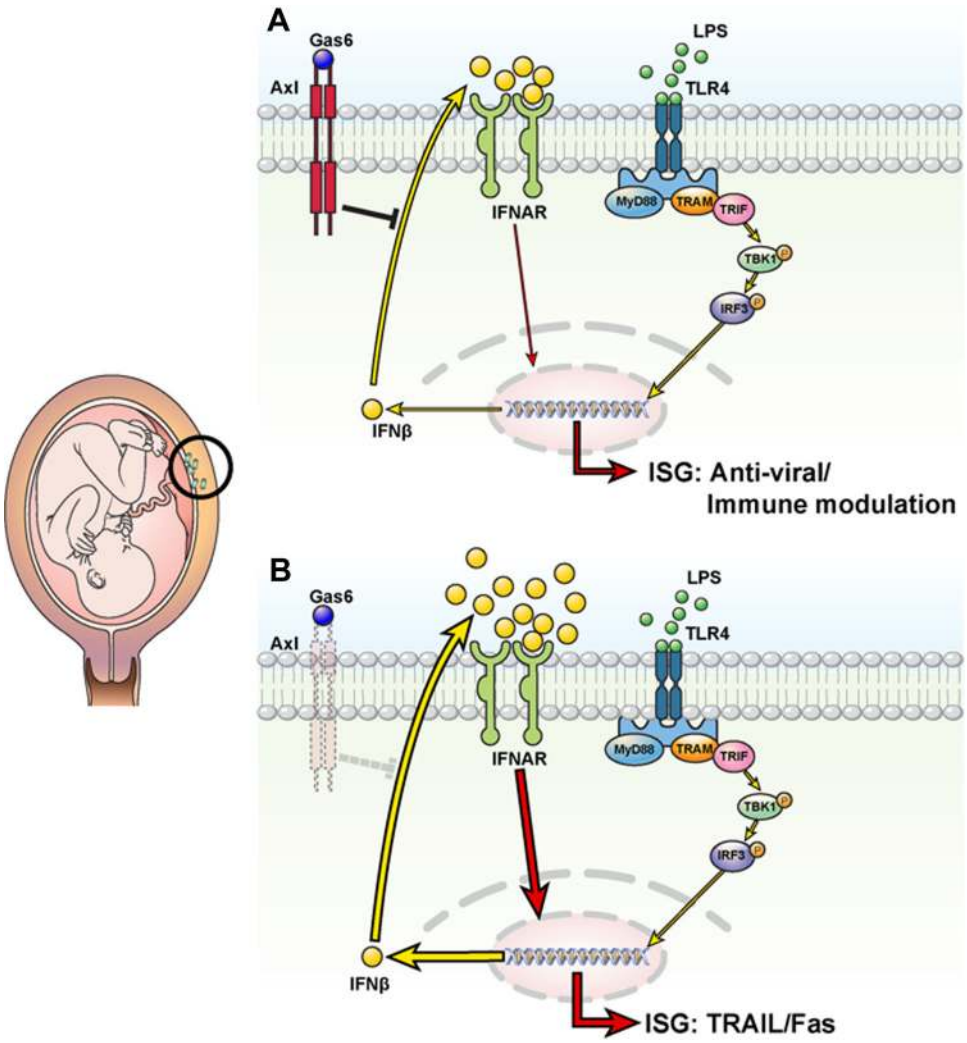


Fig. 10 Model of the regulation of IFNβ expression and function in trophoblast cells. **a** Normal regulation of IFNβ expression in trophoblast cells. LPS binds to TLR4 and activates the MyD88-independent pathway mediated by the phosphorylation of TBK and TRIF and induction of IFNβ expression. IFNβ binds to IFNAR and promotes the expression of interferon-inducible genes (ISGs). The induction of Axl and Gas6 function as negative regulators of IFNβ expression. **b** Effect of deletion of Axl on trophoblast function. In the absence of Axl expression, the negative regulation of IFNβ expression is lost, followed by enhanced IFNβ expression and selective induction of TRAIL, FAS, and ISGs regulating apoptosis

first-trimester placentas. We detected Axl and Mer expression in the trophoblast villi, but not Tyro. Similarly, isolated trophoblast expressed Axl and Mer, with higher concentrations of Axl. The in vitro studies using shRNA demonstrated that Axl was the main receptor responsible for regulating IFNβ expression since in the absence of Axl, the levels of basal as well as stimulated IFNβ were higher than in the control WT cells.

We demonstrated an effect on the regulation of basal expression of IFNβ in pregnant mice lacking Axl and Mer receptors, since TLR4 stimulation with very low concentrations (20 μg/kg) of LPS induced fetal death while the same concentration had no effect on WT pregnant mice. Interestingly, the observed fetal death was not due to a major inflammatory response, as would be the case when high doses of LPS (500 mg/kg) are administered to pregnant mice, but to increased expression of IFNβ and downstream ISGs, indicative that in addition to the role of TAM receptors in the regulation of the magnitude of IFN signaling, it also regulates the qualitative nature of IFNAR signaling. Consequently, our findings demonstrate the importance of the Axl-mediated negative feedback pathway in the regulation of IFNβ responses. The absence of such regulatory mechanisms leads to exacerbated expression of type I IFNβ, which translates into fetal demise due to the activation of IFNβ-induced placental apoptosis via TRAIL and FAS (Fig. 10 and Model Supplementary fig. 11).

These findings are in agreement with our previous report showing that viral single-stranded RNA-induced trophoblast cell death was mediated by the expression of high levels of type I IFNβ.[38] In this study, we demonstrated that in the presence of high concentrations of IFNβ, trophoblast cells underwent cell death via caspase activation. Overexpression of type I IFN in lung has been associated with an increased inflammatory response and mortality following Sendai virus infection,[49] and severe tissue damaged caused by TRAIL induces pro-apoptotic pathways in respiratory infection.[49,50] As indicated above, we observed a significant increase in TRAIL and FAS in the placentas of *AxlMer*$^{-/-}$ mice that correlated with increased IFNβ expression and apoptosis of trophoblast cells.

Poly(I:C) is a major inducer of IFNβ expression and thus can induce different ISGs.[51] Therefore, we used poly(I:C) as a model to elucidate the outcome of differential IFNβ expression and function during pregnancy. We showed that poly(I:C) could induce high levels of placental IFNβ, which also resulted in fetal demise, and

this effect was associated with the induction of pro-apoptotic ISGs. In contrast, low levels of poly(I:C) did not induce a major increase in IFNβ and consequently did not promote fetal demise, confirming that the induction of high levels of IFNβ were detrimental for fetal development. These findings are relevant since other viral infections associated with congenital complications, such as toxoplasma, rubella, cytomegalovirus, and herpes, are inducers of IFNβ expression and, if not properly regulated, may represent underlying mechanisms of the observed fetal damage.[52] Our findings demonstrate the importance of how IFNβ responses are regulated and reprogrammed during chronic infections and how alterations of its expression might have a major impact on the outcome of pregnancy.

In conclusion, we describe for the first time that (1) TLR4 ligation induces basal IFNβ expression; (2) Axl plays a critical role in regulating the expression and function of IFNβ in human and mouse trophoblast cells; (3) dysregulation of the TAM pathway results in alterations in the regulation of IFNβ/IFNAR signaling that culminate in fetal demise (Supplementary figs. 11); and (4) high levels of IFNβ and its associated ISG activation result in fetal demise. These studies underscore the essential role of IFNβ and type I IFN receptor signaling in host responses to microbial infections during pregnancy and provide novel opportunities for the identification of predictive markers and new therapeutic approaches by modulating IFNβ/TAM receptor signaling to protect pregnant women at risk for viral infections or during pandemics.

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AUTHOR CONTRIBUTIONS

J.Y.K., P.A., and K.R. conceived and performed experiments, analyzed data, and wrote the manuscript. Y.Y., J.D., X.D., J.M., G.G., and J.P. performed the experiments, V.A., M.S., L.W., and R.R. analyzed the data. GM conceived the project, analyzed the data, wrote the manuscript, and provided supervision.

ADDITIONAL INFORMATION

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