Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Precision-cut placental slices as a model to study inflammatory response

Diploma thesis

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STATEMENT OF AUTHORSHIP:

I hereby declare that I am the sole author of this diploma thesis and that I have not used any sources other than those listed in the bibliography and identified as references. I further declare that I have not submitted this thesis at any other institution to obtain a degree.

In Hradec Králové Bahareh Bolboli

Date: 15.08.2023

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ABSTRACT

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Title of the diploma thesis: Precision-cut placental slices as a model to study inflammatory response.

Maternal inflammation during pregnancy is a recognized factor linked to an array of complications and potential neurodevelopmental and neuropsychiatric risks for offspring. Placenta is the crucial interface between maternal and fetal domains. This organ not only shapes fetal development but also possesses the ability to respond to inflammatory stimulations, potentially in a sex-specific manner. Nonetheless, the fundamental immunoregulatory mechanisms orchestrating such responses remain unclear. To bridge this knowledge gap, our study harnessed an innovative approach: the ex vivo precision-cut placental slice model using rat placentas. This experimental design was tailored to probe acute inflammatory responses.

Precision-cut placental slices, precisely 200 µm thick, were meticulously generated from both male and female rat placentas. These slices were subjected to varying concentrations of Lipopolysaccharide (LPS) or Polyinosinic: polycytidylic acid (Poly I:C) for discrete periods of 4 and 18 hours. Our investigative journey uncovered compelling revelations: LPS stimulation triggered a robust upswing in the expression and subsequent release of proinflammatory cytokines, specifically Tumor necrosis factor α (TNF-α), Interleukin 6 (IL-6), and Interleukin 1β (IL-1β). In stark contrast, Poly I:C exposure elicited a more subdued inflammatory reaction. A remarkable twist emerged as the female placenta, when challenged by LPS, unveiled heightened sensitivity compared to its male counterpart. In summary, in this diploma thesis we introduce the rat placental slices as an avant-garde experimental model, effectively unlocking

the realm of sexual dimorphism within acute inflammatory responses and immune activity during pregnancy. Within this model, there is the potential to unravel the complex interactions connecting maternal inflammation, placental functionality, and fetal outcomes, thereby casting light upon prospective therapeutic pathways.

ABSTRAKT

Univerzita Karlova

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Název diplomové práce: Placentární řezy jako model pro studium zánětlivé odpovědi.

Zánět probíhající v těle matky je rozpoznaným faktorem spojeným s řadou komplikací a potenciálními riziky pro neurovývoj a neuropsychiatrické faktory potomků. Centrálním prvkem této dynamiky je placenta, klíčové rozhraní mezi mateřským a plodovým prostředím. Tento orgán nejen formuje vývoj plodu, ale má také schopnost reagovat na zánětlivé podněty, specifickým způsobem závislosti na pohlaví plodu. Nicméně základní imunoregulační mechanismy, které řídí takové reakce, jsou neznámé. Pro překonání tohoto znalostního deficitu využila naše studie inovativní přístup: ex vivo model přesného placentárního řezu za použití placenty potkana na konci březosti. Tento experimentální návrh byl vytvořen pro zkoumání akutních zánětlivých reakcí.

Precizně řezané placentární preparáty o tloušťce přesně 200 μm byly pečlivě vytvořeny jak z placent samců, tak i samic potkanů. Tyto preparáty byly vystaveny různým koncentracím lipopolysacharidu (LPS) nebo Polyinosinické: polycytidylové kyseliny (Poly I:C) po dobu 4 až 18 hodin. Náš způsob výzkumu objevil zajímavá data: stimulace LPS vyvolala robustní vzestup v expresi a následném uvolnění prozánětlivých cytokinů, a to konkrétně faktoru nádorové nekrózy α (TNF-α), Interleukinu 6 (IL-6) a Interleukinu 1β (IL-1β). Na druhé straně vystavení Poly I:C vyvolalo mírnější zánětlivou reakci. Samičí placenta, konfrontována s LPS, prokázala zvýšenou citlivost ve srovnání se samčí placentou.

Naše studie představuje placentární řezy potkana jako inovativní experimentální model, který efektivně otevírá oblast pohlavního dimorfismu v rámci akutních zánětlivých reakcí a imunitních aktivací během těhotenství. V tomto modelu spočívá potenciál k rozluštění složitého vzájemného působení mezi mateřským zánětem, funkčností placenty a výsledky pro plod, a tím osvětluje budoucí terapeutické možnosti.

CONTENTS

1 ABBREVIATIONS

LPS – Lipopolysaccharide

Poly I:C – Polyinosinic: polycytidylic acid

LDH – Lactate dehydrogenase

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethylsulfoxide

FBS – Fetal bovine serum

qPCR – quantitative polymerase chain reaction

β2m – β-2 microglubulin

Tbp – TATA-binding protein

Ywhaz – Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

TNF-α – Tumor necrosis factor-α

IL-6 – Interleukin-6

IL-10 – Interleukin-10

IL-1β – Interleukin-1β

Tlr4 – Toll-like receptor 4

Tlr3 – Toll-like receptor 3

ELISA – enzyme-linked immunosorbent assay

NF-κB – Nuclear factor Kappa B

cDNA – complementary DNA

mRNA – messenger ribonucleic acid

RNA – ribonucleic acid

2 INTRODUCTION

The placenta, an extraordinarily transient organ, plays a myriad of crucial roles in facilitating the exchange of gases, nutrients, and waste products between the maternal and fetal circulations. Beyond its pivotal functions in mediating these exchanges, the placenta also serves as a remarkable factory, generating vital metabolites and hormones essential for optimal fetal growth and development. Additionally, it acts as a formidable guardian, establishing an immunological barrier that shields the developing fetus from potential threats posed by the maternal immune system (Jansson and Powell 2007, Costa 2016, Knöfler, Haider et al. 2019, Cindrova-Davies and Sferruzzi-Perri 2022). Throughout the intricate process of gestation, the placenta undergoes a symphony of dynamic physiological and structural changes, meticulously choreographed to ensure the precise orchestration of fetal development and programming. Disruptions in the intricate processes of placental formation and function triggered by various external insults, including inflammation, pharmacotherapy, malnutrition, and environmental signals, have the potential to give rise to pregnancy complications and exert a profound impact on fetal programming (Jansson and Powell 2007, Cindrova-Davies and Sferruzzi-Perri 2022).

Maternal inflammation has emerged as a critical factor implicated in a wide spectrum of pregnancy pathologies, encompassing preterm birth, pre-eclampsia, and intrauterine growth restriction. Moreover, it represents a significant risk factor for neurodevelopmental and neuropsychiatric disorders in offspring (Patterson 2009, Cotechini and Graham 2015). These pregnancy-related disorders are characterized by an intricate interplay of elevated levels of proinflammatory cytokines found within both maternal serum and the placental microenvironment (Patterson 2009, Cotechini and Graham 2015, Yockey and Iwasaki 2018). The activation of proinflammatory cytokines, including TNF-α, IL-6, IL-1β, and Interferon γ (IFN-γ), in response to maternal inflammation, contributes to the heightened vulnerability of neuropsychiatric disorders during crucial periods of fetal development. While cytokines play indispensable roles in normal fetal development, dysregulated levels can give rise to a spectrum of developmental defects affecting multiple organs, including the placenta, brain, heart, and lungs (Yockey and Iwasaki 2018). This dysregulation primarily occurs through the intricate process of direct transplacental transfer of maternal cytokines (Zaretsky, Alexander et al. 2004, Ratnayake, Quinn et al. 2013). In this intricate scenario, proinflammatory cytokines have surfaced as pivotal molecular orchestrators, modulating various pathways within the placenta, including those involved in the development of dopamine- and serotonin-dependent neurogenic

pathways and tryptophan metabolism (Goeden, Velasquez et al. 2016, Aguilar-Valles, Rodrigue et al. 2020, Karahoda, Robles et al. 2021). Intriguingly, emerging evidence suggests that the placental response to maternal inflammation is influenced by the fetal sex, although conflicting findings exist within the published literature (Sood, Zehnder et al. 2006, Cai, van Mil et al. 2016, Braun, Carpentier et al. 2019). Hence, further dedicated research endeavors are warranted to unravel the intricate molecular mechanisms underlying the placental immune response to inflammation and delineate the nuanced role played by fetal sex in shaping this complex interplay.

Recent advances in research have unveiled the immense potential of precision-cut slices as a powerful tool for investigating inflammatory responses within various tissues, ranging from the intricate pulmonary architecture to the multifaceted hepatobiliary network and the elaborate intestinal milieu (Olinga, Merema et al. 2001, Sauer, Vogel et al. 2014, Li, de Graaf et al. 2016, Liu, Särén et al. 2021, Hasuda, Person et al. 2022). Despite these advancements, the application of this promising technique in elucidating acute inflammatory responses within the placenta remains a relatively uncharted territory. Precision-cut placental slices embody a unique advantage, encompassing the vast tapestry of cell types that characterize the placental microenvironment, while faithfully preserving the intricate three-dimensional architecture that governs its function. Consequently, these slices provide an invaluable platform for studying the sexually dimorphic (patho)physiological processes that underlie placental function (Gilligan, Tong et al. 2012). Considering the striking similarities in structural characteristics shared between the human and rat placenta, owing to their shared hemochorial nature, the rat placenta emerges as an optimal model for deciphering the intricacies of placental physiology. Building upon this premise, our study ventures forth with the proposition that the ex vivo cultivation of precision-cut slices derived from the rat placenta presents an innovative and compelling alternative approach for interrogating sex-dependent inflammatory responses and exploring the multifaceted landscape of inflammation-associated pathologies entrenched within the placenta (Figure 1). To elicit inflammation, we harnessed the potential of both bacterial agents, such as lipopolysaccharide (LPS), and viral agents, exemplified by Polyinosinic: polycytidylic acid (Poly I:C). By incorporating this multifaceted experimental design, we aim to discern the impact of inflammation on various facets, including i) the viability and integrity of placental tissues, ii) the intricate landscape of proinflammatory genes and proteins within male and female placentas.

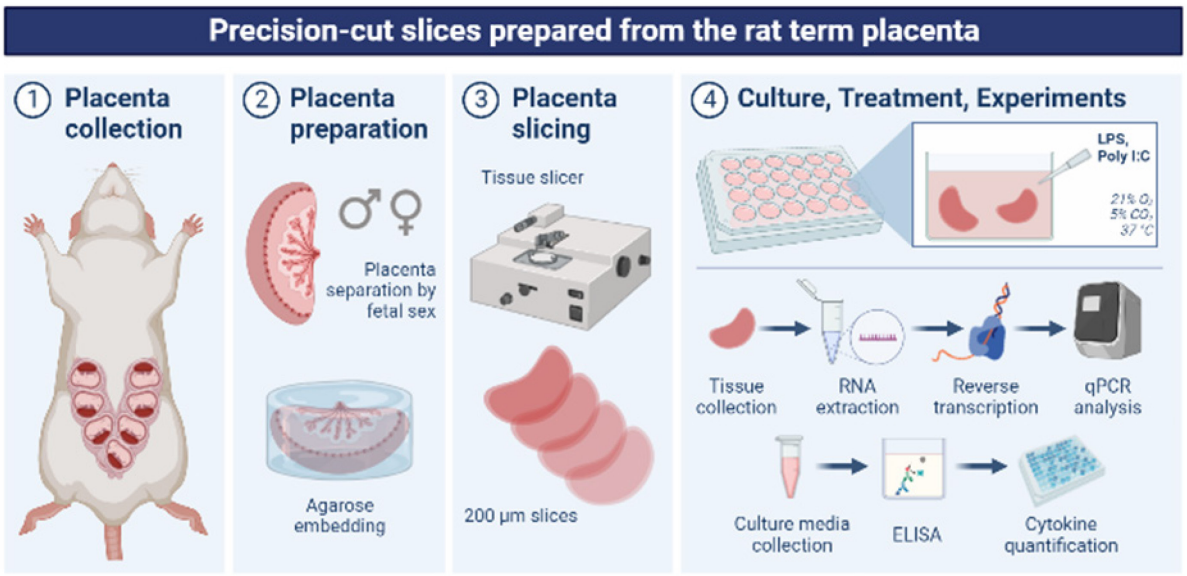

Figure 1. This figure legend provides an overview of the experimental workflow utilized to investigate acute inflammatory responses in the placental slices. On twentieth gestation day, rats were subjected to anesthesia, and abdominal midline incisions were made to expose. Gestational sacs were carefully isolated, and placental samples were segregated based on fetal gender. The entire placental tissue was encased within agarose, and precision-cut placental slices with a uniform thickness of 200 µm were generated utilizing a sterilizable microtome. These placental slices were cultured in 24-well plates for a stabilization period of 36 hours. Subsequently, they were exposed to various concentrations of lipopolysaccharide (LPS) (0.1, 1, 5 µg/ml) or Polyinosinic: polycytidylic acid (Poly I:C) (1, 10, 50 µg/ml) for 4 and 18 hours. Following the treatment, both tissue samples and culture media were collected for quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA) measurements, respectively (Anandam, Abad et al. 2022).

3 BACKGROUND

Placenta

The placenta, an intricately organized and pregnancy-specific organ, assumes a pivotal role in orchestrating fetal development and maintaining gestation. Structurally and functionally sophisticated, the placenta serves as a vital hemochorial interface, facilitating intricate bidirectional exchanges of gases, nutrients, and metabolic waste products between the maternal and fetal circulatory systems (Figure 2). Additionally, it acts as a multifaceted endocrine gland, synthesizing and secreting an array of hormones crucial for the regulation of pregnancy and promotion of fetal growth (Elad, Levkovitz et al. 2014, Staud and Karahoda 2018, Furukawa, Tsuji et al. 2019, Ortega, Fraile-Martínez et al. 2022). While much of our understanding of placental biology comes from human studies, animal models provide valuable insights into placental development and function. Among these models, the rat placenta has emerged as a useful tool due to its striking structural and functional similarities to the human placenta. The rat placenta shares a hemochorial organization, where fetal chorionic villi are in direct contact with maternal blood, facilitating efficient nutrient and gas exchange (Maranghi, Macrì et al. 1998, Furukawa, Kuroda et al. 2014, Furukawa, Tsuji et al. 2019).

In terms of anatomical structure, the rat placenta exhibits chorionic villi that are analogous to those found in the human placenta. These villi project into the maternal decidua basalis, ensuring an extensive surface area for effective maternal-fetal exchange. The placental barrier in rats, like in humans, consists of layers of trophoblast cells and maternal endothelial cells, enabling selective transfer of substances while providing protection against pathogens (Staud and Karahoda 2018, Furukawa, Tsuji et al. 2019).

Furthermore, the rat placenta demonstrates functional similarities to the human placenta. It is involved in the transport of gases, nutrients, and waste products between the maternal and fetal circulations. Similar to the human placenta, the rat placenta orchestrates the transfer of oxygen from maternal blood to fetal circulation, ensuring adequate oxygenation for fetal development. Carbon dioxide and metabolic waste products are also eliminated from the fetal to the maternal bloodstream (Staud and Karahoda 2018, Furukawa, Tsuji et al. 2019).

In addition to nutrient and gas exchange, the rat placenta performs endocrine functions akin to the human placenta. It synthesizes and secretes hormones such as progesterone, estrogens, and lactogens, which are crucial for maintaining pregnancy, supporting fetal growth, and preparing

the maternal body for lactation. Research utilizing the rat placenta has provided valuable insights into the impact of various factors on placental development and function. Investigations on maternal inflammation, pharmacotherapy, malnutrition, and environmental signals have shed light on how these external insults can perturb placental formation and function, leading to pregnancy complications and affecting fetal programming. Understanding the similarities between the rat and human placenta allows researchers to utilize the rat model to explore the underlying mechanisms of placental biology, study the effects of interventions, and gain insights into human placental function and pathologies. The rat placenta's amenability to experimental manipulation, combined with its similarities to the human placenta, positions it as a valuable tool for advancing our understanding of placental physiology and developing strategies to improve maternal and fetal health outcomes (Furukawa, Hayashi et al. 2011, Charest, Vrolyk et al. 2018, Staud and Karahoda 2018).

While human studies provide invaluable insights into placental biology, the rat placenta serves as a valuable model due to its structural and functional similarities. The rat placenta shares a hemochorial organization, exhibits analogous anatomical structures, and performs comparable functions to the human placenta (Furukawa, Tsuji et al. 2019). Utilizing the rat model allows researchers to investigate placental development, function, and the impact of external insults on pregnancy outcomes. By leveraging the similarities between the rat and human placenta, we can further our understanding of placental biology and translate these findings to improve maternal and fetal health (Charest, Vrolyk et al. 2018).

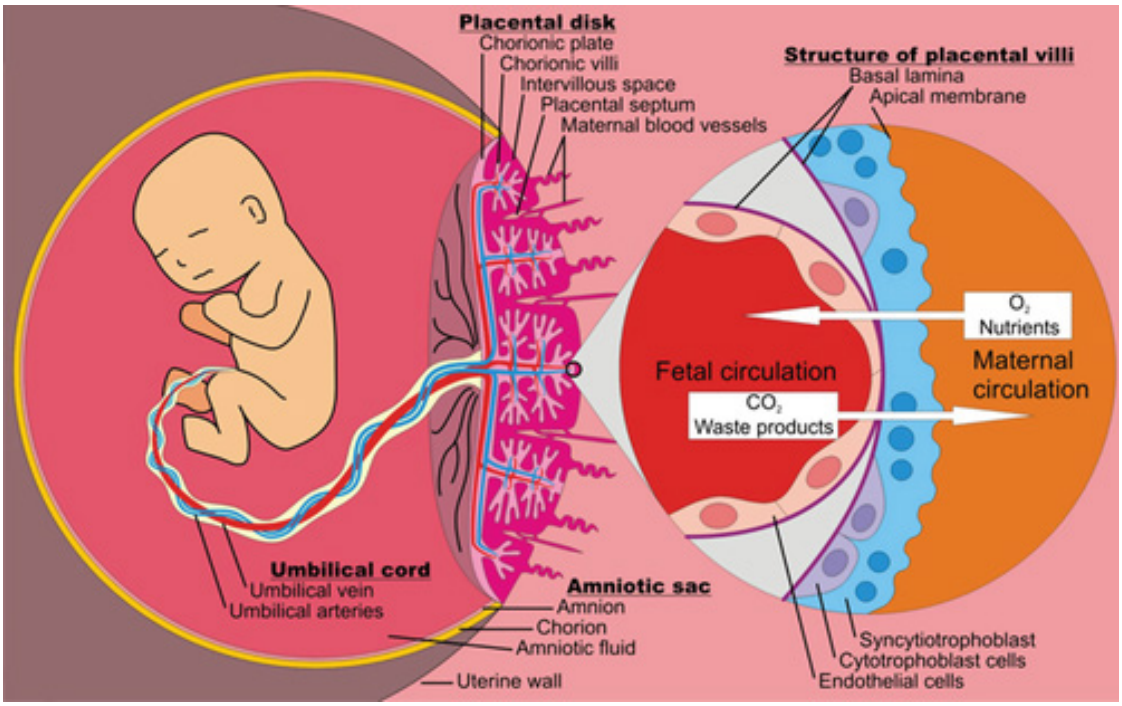

Figure 2. The placenta plays a crucial role as a barrier, facilitating the supply of oxygen and essential nutrients to the fetus (Elad, Levkovitz et al. 2014).

Inflammation

Inflammation is a fundamental biological response that plays a crucial role in the body's defense against infection, injury, and tissue damage. It involves complex interactions between immune cells, signaling molecules, and various mediators. Cytokines and interleukins, a subset of inflammatory mediators, play a central role in coordinating and regulating the inflammatory response (Panigrahy, Gilligan et al. 2021, Roe 2021). Cytokines are small proteins or peptides secreted by immune cells, as well as other cell types including trophoblasts, in response to infection, injury, or immune stimulation. They act as powerful signaling molecules that mediate communication between cells and orchestrate immune responses. Cytokines are involved in a wide range of biological processes, including cell activation, proliferation, differentiation, migration, and inflammation regulation. Interleukins, a specific class of cytokines, are produced by immune cells such as macrophages, lymphocytes, and dendritic cells. They play a critical role in modulating immune responses and is involved in pro-inflammatory pathway. Interleukins, such as IL-1β, IL-6, and IL-10, have diverse effects on immune cells and can either promote or suppress inflammation depending on the context (Roe 2021).

During the inflammatory process, immune cells are activated and recruited to the site of infection or tissue damage. Neutrophils are among the first responders, followed by monocytes and macrophages. These cells release pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, which initiate and propagate the inflammatory cascade. Pro-inflammatory cytokines promote the dilation and permeability of blood vessels, facilitating the migration of immune cells to the site of inflammation. They activate endothelial cells to express adhesion molecules that facilitate leukocyte adhesion and extravasation. In turn, leukocytes release additional cytokines, amplifying the inflammatory response (Roe 2021).

Placental inflammation can have significant implications for both maternal and fetal health. Maternal inflammation during pregnancy has been associated with pregnancy pathologies such as preterm birth, preeclampsia, and intrauterine growth restriction, as well as an increased risk of neurodevelopmental and neuropsychiatric disorders in offspring (Figure 3). Proinflammatory cytokines play a key role in mediating the adverse effects of inflammation on

placental function and fetal development (Goldstein, Gallagher et al. 2020, Karahoda, Robles et al. 2021, Anandam, Abad et al. 2022, Ward, Bert et al. 2022).

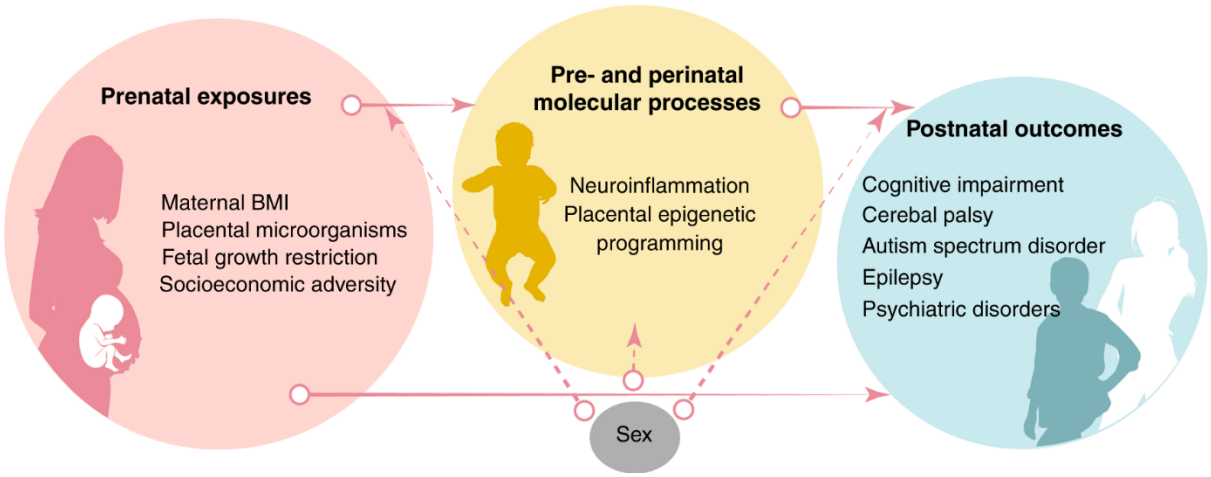

Figure 3. In this figure we can see connection of early life risk factors and the foundational mechanisms that contribute to differences in neurodevelopment among individuals later in life (Bangma, Hartwell et al. 2021).

Precision-cut slices

Ex vivo precision-cut slices have emerged as a valuable tool in biomedical research for studying tissue responses and pathophysiological processes in a controlled environment. This technique involves the preparation of thin tissue slices, typically ranging from 100 to 500 micrometers in thickness, that retain the structural and functional integrity of the tissue. The use of *ex vivo* cultured precision-cut slices offers several advantages over other experimental approaches. By preserving the tissue's native architecture and cellular organization, precision-cut slices provide a more accurate representation of in vivo conditions (Gilligan, Tong et al. 2012, Nizamoglu, Joglekar et al. 2023).

Moreover, precision-cut slices maintain the tissue's three-dimensional structure, including the intricate cellular networks, and extracellular matrix. This preservation enables the examination of cell-cell and cell-matrix interactions, as well as the spatial distribution of molecules or markers within the tissue (Dewyse, Reynaert et al. 2021, Fahmi, Brügger et al. 2022).

The ex vivo nature of precision-cut slices allows for the precise control of experimental conditions and the elimination of confounding factors present in in vivo models. Researchers

can manipulate and assess specific variables, such as the exposure to inflammatory stimuli or the effects of therapeutic interventions, with high precision (Olinga, Merema et al. 2001, Sauer, Vogel et al. 2014).

Precision-cut slices also provide a platform for studying dynamic processes and time-dependent responses. The slices can be cultured ex vivo over a defined period, allowing for the assessment of acute and chronic effects. This temporal control enables the investigation of the kinetics of tissue responses and the progression of cellular processes. Ex vivo cultured precision-cut slices have proven to be valuable tools in the field of pharmacology, toxicology, physiology and disease modeling (Olinga, Merema et al. 2001, Sauer, Vogel et al. 2014, Li, de Graaf et al. 2016, Ruigrok, Maggan et al. 2017). They have been used to evaluate drug metabolism, study toxic effects and investigate disease mechanisms.

Researchers can employ ex vivo cultured precision-cut slices from lungs, liver, intestine, and kidney to explore the effects of external stimuli, such as bacterial or viral agents, on placental tissue viability, inflammatory cytokine production, and other relevant markers. This technique facilitates the assessment of sex-dependent responses, the exploration of specific pathways affected by inflammation. However, very limited research was performed on placental slices and there has been no attempt made to explore inflammatory response using placental precision-cut slices (Poosti, Pham et al. 2015, Li, de Graaf et al. 2016, Ruigrok, Maggan et al. 2017, Liu, Särén et al. 2021).

Overall, ex vivo precision-cut slices provide a powerful experimental approach for studying tissue responses and pathophysiological processes. Their ability to preserve tissue structure, enable precise experimental control, and offer a controlled ex vivo environment makes them particularly valuable in understanding complex biological phenomena. In the field of placental research, ex vivo precision-cut slices allow for the investigation of inflammation and its effects on placental function, contributing to our understanding of pregnancy-related pathologies and potential therapeutic interventions.

4 AIM OF STUDY

The aim of the study was to generate and validate a novel experimental model of precision-cut rat placental slices to investigate and explore the inflammatory responses within the placental microenvironment. Specifically, the study sought to understand how bacterial (LPS) and viral (Poly I:C) infection model activates inflammatory response in placental slices and examine potential sexual dimorphic effects in these immune responses. The specific aims were to:

1. Validate the viability and integrity of the placental slices exposed to LPS and Poly I:C.
2. Analyze the gene expression of pro-inflammatory cytokines and Toll-like receptors in placental slices exposed to LPS and Poly I:C.
3. Evaluate the secretion of pro-inflammatory cytokines in placental slices exposed to LPS and Poly I:C.

5 MATERIALS AND METHODS

Reagents

LPS from Escherichia coli O111:B4 (product #: L2630), Poly I:C (product #: P1530), Thiazolyl blue tetrazolium bromide (MTT; product #: M5655), Gentamycin (product #: G1397) were purchased from Sigma-Aldrich (St. Louise, MO, USA), Tri Reagent solution was obtained from the Molecular Research Centre (Cincinnati, OH, USA). All other chemicals were of analytical grade.

Animals

It is important to note that the choice of an animal model depends on the specific research question, goals, and ethical considerations. Wistar rats are widely recognized and commonly used as an animal model in scientific research for several reasons. Their suitability as a model organism stems from various characteristics and advantages that make them valuable for studying a wide range of biological processes and diseases. Here are some reasons why Wistar rats are considered a good model for scientific investigations they have genetic stability, availability, standardization and adaptability, plus their size and physiology is very good for studying disease models and finally, the ethical considerations.

Pregnant Wistar rats (10-14 weeks old) were purchased from Velaz, Ltd. (Czech Republic). The animals were kept in cages at constant room temperature, low noise and standard conditions of 12L: 12D. All rats received food and water ad libitum. Day 1 of pregnancy (Gd1) was proven by the presence of a sperm clump after night mating. In Gd 20, rats were anesthetized with pentobarbital with a dose of 40 mg/kg to the tail vein. The pregnancy sacs were separated and 4 males and 4 females (determined by measuring the anogenital distance in the fetuses optically) placenta were collected separately from each dam in the Krebs-Henseleit buffer [prepared as previously described] (de Graaf, Olinga et al. 2010). “n” represents the number of dams. The experiments were approved by the Ethics Committee of the Faculty of Pharmacy of Hradec Kralove (University of Charles in the Czech Republic) and conducted in accordance with the Laboratory Animal Care and Use Guide 1996 and the European Convention on the Protection of Biological Animals for Experimental and Other Scientific Activities

Preparation and culture of ex vivo rat placenta slices

Placental slicing (Figure 4) is performed using the Gilligan et al., protocol (Gilligan, Tong et al. 2012). The entire placenta is incorporated with 3% low-gelling agarose and the agarose temperature is maintained between 36.5°C and 37 ° C to avoid destruction of the placenta tissue. Once solidified, the agarose embedded placenta was transferred to the Krumdiec tissue slicer (Alabama R&D, Munford, AL, USA), and a slice of the placenta was generated with a precise thickness of 200 µm (moderate speed; 4 slices/minute).

Approximately 40-50 mg of intact slices were cultured in 24-well plates (TPP, Switzerland) containing 1 ml of Dulbecco modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 µg/ml gentamycin. Slices were cultured under normal cell culture conditions, 21% O^2, 5% CO^2 at 37 °C, and fresh medium was replaced every 24 hours.

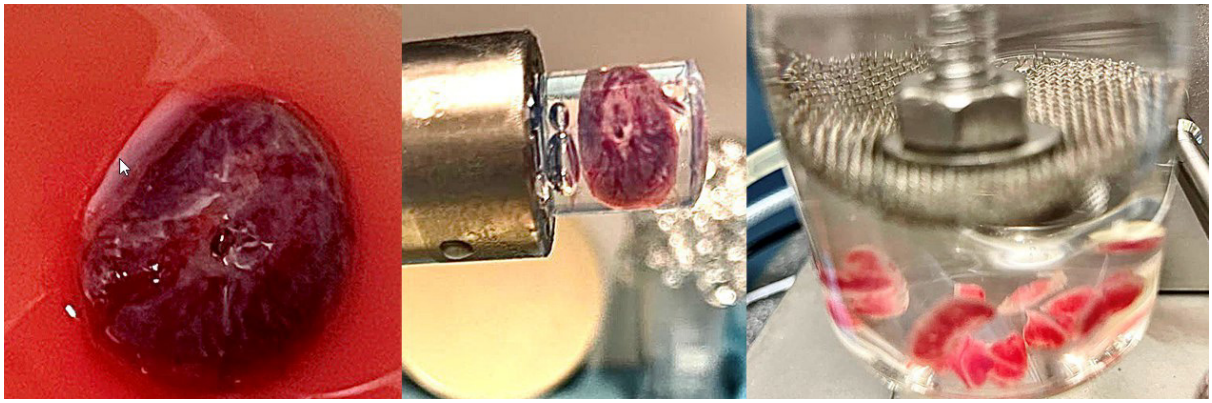

Figure 4. Process of rat placental precision-cut slicing.

Treatment of placenta slices with LPS and Poly I:C

Placental slices were cultivated for 36 hours under normal conditions (stabilization period) and exposed for 4 and 18 hours to different concentrations of LPS (0.1, 1, 5 µg/ml) or poly I:C (1, 10, 50 µg/ml). After treatment, the tissues and the culture medium were collected, and stored at -80°C until further analysis.

Lactate dehydrogenase Activity

Lactate dehydrogenase (LDH) is an enzyme found in all cell types, including placental cells, and plays a vital role in cellular metabolism. Measuring LDH activity is commonly used to assess cell damage or cytotoxicity. In the context of precision-cut placental slices, LDH activity serves as an indicator of tissue integrity. By quantifying LDH release into the culture media, researchers can evaluate the integrity of the slices.

The integrity of the placental slices was tested by measuring LDH release into the culture media using a colorimetric LDH activity assay kit (product #: MAK066; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm in a 96-well plate using the Hidex Sense β Plus microplate reader (Hidex, Finland). LDH activity in the media was normalized to the tissue weight.

MTT assay

MTT assay is a commonly used method to assess cell viability and metabolic activity. It relies on the ability of viable cells to convert the yellow water-soluble MTT compound into purple formazan crystals by mitochondrial enzymes. The amount of formazan formed is directly proportional to the number of viable cells present.

To evaluate the incorporation of MTT into the tissue, placental slices were incubated with 1 mg/ml of MTT (in phenol red-free medium) at 37° C for 2 hours. Subsequently, the slices were placed in Dimethylsulfoxide (DMSO) and incubated for 5 minutes to release the formazan crystals. The absorbance was measured at 570 nm and 690 nm using the Hidex Sense β Plus microplate reader (Hidex, Finland). Results are expressed as the difference between 570 and 690 nm absorbance and normalized to the tissue weight.

RNA extraction and quantitative polymerase chain reaction analysis

RNA extraction is the first step in gene expression assays using different technologies such as reverse transcription-quantitative polymerase chain reaction (RT-qPCR), RNA sequencing, and microarray.

Total RNA was extracted from the placental slices using TRI reagent (Molecular Research Centre, Cincinatti, Ohio, United States) according to the manufacturer's instructions were: The collected placenta samples were subjected to homogenization, a process where the tissue is

mechanically or chemically broken down into smaller particles. This step aims to release the cellular components, including RNA, from the tissue structure. After homogenization, the sample is subjected to a phase separation step. This typically involves the addition of certain reagents that cause the sample to separate into distinct phases, such as an aqueous phase (containing RNA) and an organic phase (Figure 5). This separation helps isolate the RNA from other cellular components. In this step, specific reagents are added to the aqueous phase to induce the precipitation of RNA molecules. This causes the RNA to clump together and separate from the solution. The precipitated RNA is carefully washed with appropriate solutions. This step is crucial to remove impurities, contaminants, and residual reagents that might interfere with downstream analyses. Following washing, the purified RNA pellet obtained from the precipitation is solubilized in a suitable buffer or solution. This makes the RNA molecules available in a dissolved form, ready for further analysis. This step is essential to ensure that the RNA is in a state suitable for accurate quantification or downstream experiments like reverse transcription for complementary DNA(cDNA) synthesis. Throughout these steps, maintaining a sterile and controlled environment is vital to prevent contamination. Each stage contributes to isolating high-quality RNA, which is crucial for obtaining reliable and meaningful results in subsequent molecular analyses.

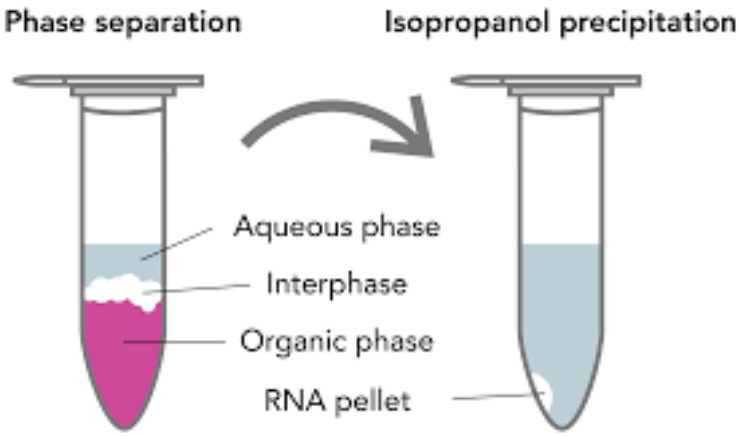

Figure 5. Phase separation in RNA extraction. Adopted from Zymo Research

The concentration and purity of RNA were determined using a NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized using the iScript Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the kit protocol. cDNA was synthesised from an RNA template in a reaction catalysed by the enzyme Reverse Transcriptase (RTase). In order to initiate gene amplification, a short DNA oligonucleotide called a primer is needed to anneal the template cDNA. The amplification of inflammatory genes was performed in triplicate using the Taqman^{TM} Universal Master Mix II (Thermo Fisher Scientific, Waltham, MA, United States). The sample was amplified to 5µl (384 well plate format) reaction volume according to the manufacturer's recommended thermal conditions (Figure 6). Target gene expression was calculated using the ΔΔCT method (23), and relative expression was normalized with the geometric mean of β-2 microglobulin (β2m), TATA binding protein (Tbp) and activation protein tyrosine 3-monooxygenase / tryptophan-5-monooxygenase (Ywhaz). The following primers were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and used in our study. Tnf-α (Rn99999017_m1), IL-6 (Rn01410330_m1), IL-1β (Rn00580432_m1), Tlr3 (Rn01488472_g1), Tlr4 (Rn00569848_m1), Ywhaz (Rn00755072_m1), β2m (Rn00560865_m1) and Tbp (Rn01455646_m1).

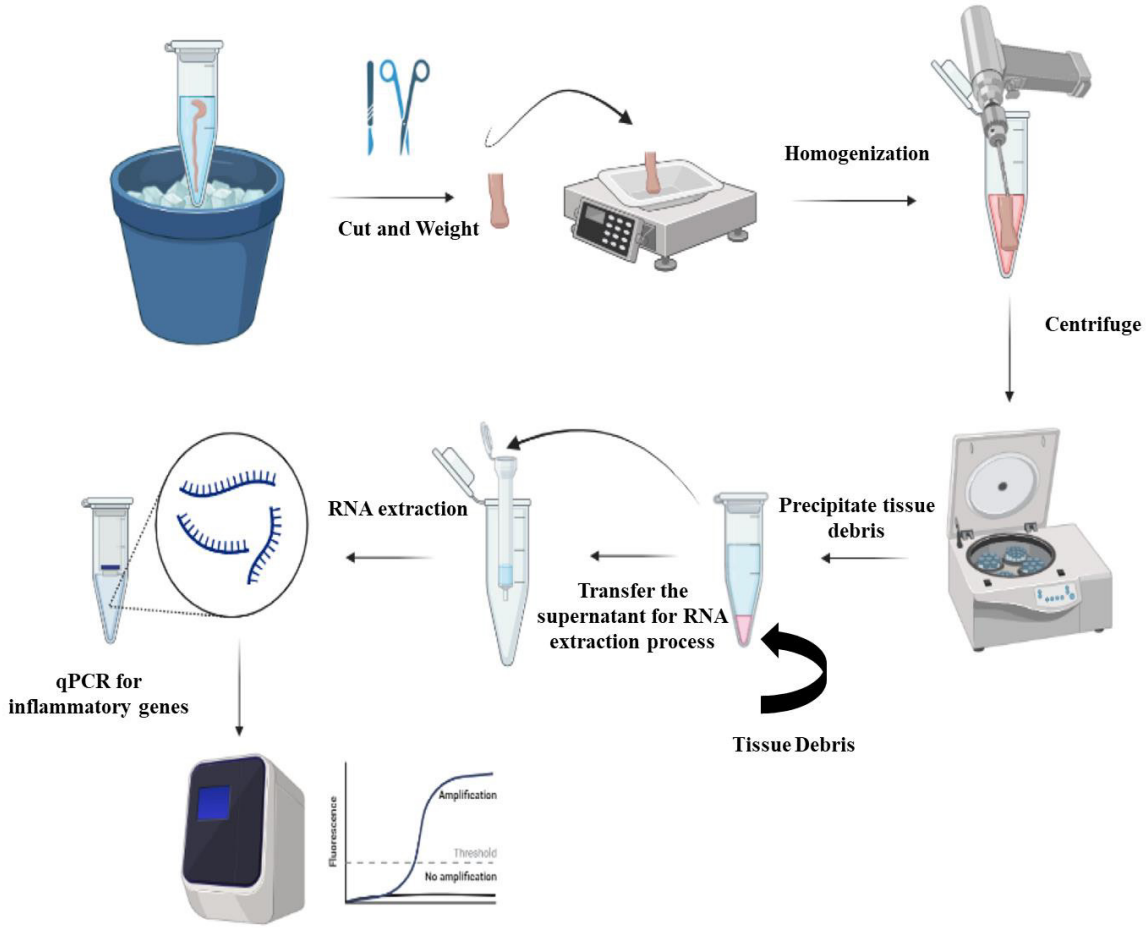

Figure 6. Steps of RNA extraction and quantitative polymerase chain reaction analysis (Sayed, Inouye et al. 2023).

Cytokine quantification by ELISA

ELISA (Enzyme-Linked Immunosorbent Assay) is a widely used technique in biomedical research to detect and quantify specific proteins or molecules of interest. The sandwich ELISA, in particular, is a common format employed to measure the concentration of target proteins in biological samples (Figure 7). This assay format involves the use of capture and detection antibodies that bind to different epitopes on the target protein, forming a "sandwich" complex. The captured protein is subsequently detected using a biotin streptavidin-conjugated secondary antibody and a substrate that produces a measurable signal.

In accordance with manufacturer instructions, the concentration of TNF-α (CATALOG: ERA56RB), IL-6 (CATALOG: ERA31RB), and IL1-β (CATALOG: BMS630) were measured in a cell-free culture medium using a commercially available ELISA kit from Thermo Fisher Scientific (Waltham, Massachusetts). The concentration of cytokine in the media was normalized according to the weight of the tissue.

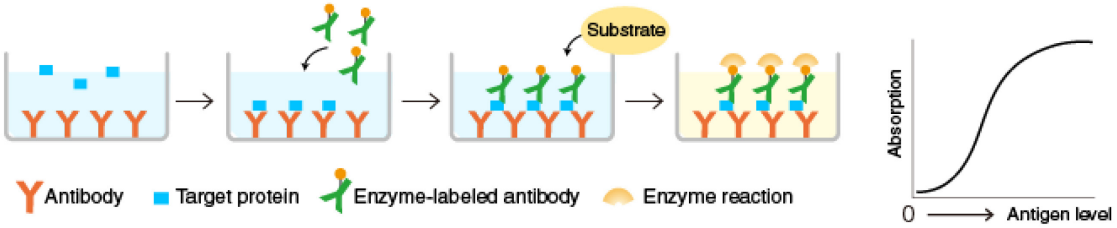

Figure 7. Sandwich ELISA principle. Adopted from MBL life science

Statistical analysis

Statistical analyses were implemented in GraphPad Prism 8.3.1 software (GraphPad Software, Inc., San Diego, USA) using two-way ANOVA followed by Dunnett's multiple comparison tests. All experiments were performed in 3-5 biological and 3 technical replicates. The n number represents the number of dams from which 4 male and 4 female placentas were

collected to generate the slices. Data are presented as mean ± SEM and results were considered significant when $p < 0.05$.

6 RESULTS

Viability and integrity of placental slices during cultivation and treatment

Viability Assessment.

The viability of the rat placental slices was evaluated using the MTT assay, which provides a measure of metabolic activity indicative of cell viability. The MTT assay results revealed that the viability of the placental slices remained unchanged for up to 120 hours of culture (Figure 8A), indicating their sustained viability throughout the experimental duration.

Integrity Assessment.

The integrity of the placental slices was assessed by quantifying the release of LDH into the culture media. LDH activity serves as an indicator of cellular damage or compromise. After 24 hours of culture, the LDH activity reached approximately 400 mU/ml/g and gradually declined to baseline levels (< 50 mU/ml/g) thereafter, remaining stable for the entire 120 hour culture period (Figure 8B). These findings demonstrate the preserved structural integrity of the placental slices throughout the experiment.

Cytotoxicity effect of LPS and Poly I:C on placental slices.

To determine the potential cytotoxic effects of LPS and Poly I:C treatment on the placental slices, LDH activity was measured in samples treated for 4 and 18 hours. Intriguingly, no significant increase in LDH release was observed in the culture media of LPS/Poly I:C-exposed slices compared to the control group (Figures 8 C-F). These results suggest that the placental slices maintained their viability and did not experience substantial cellular damage when exposed to the tested concentrations and durations of LPS and Poly I:C in both male and female placental slices.

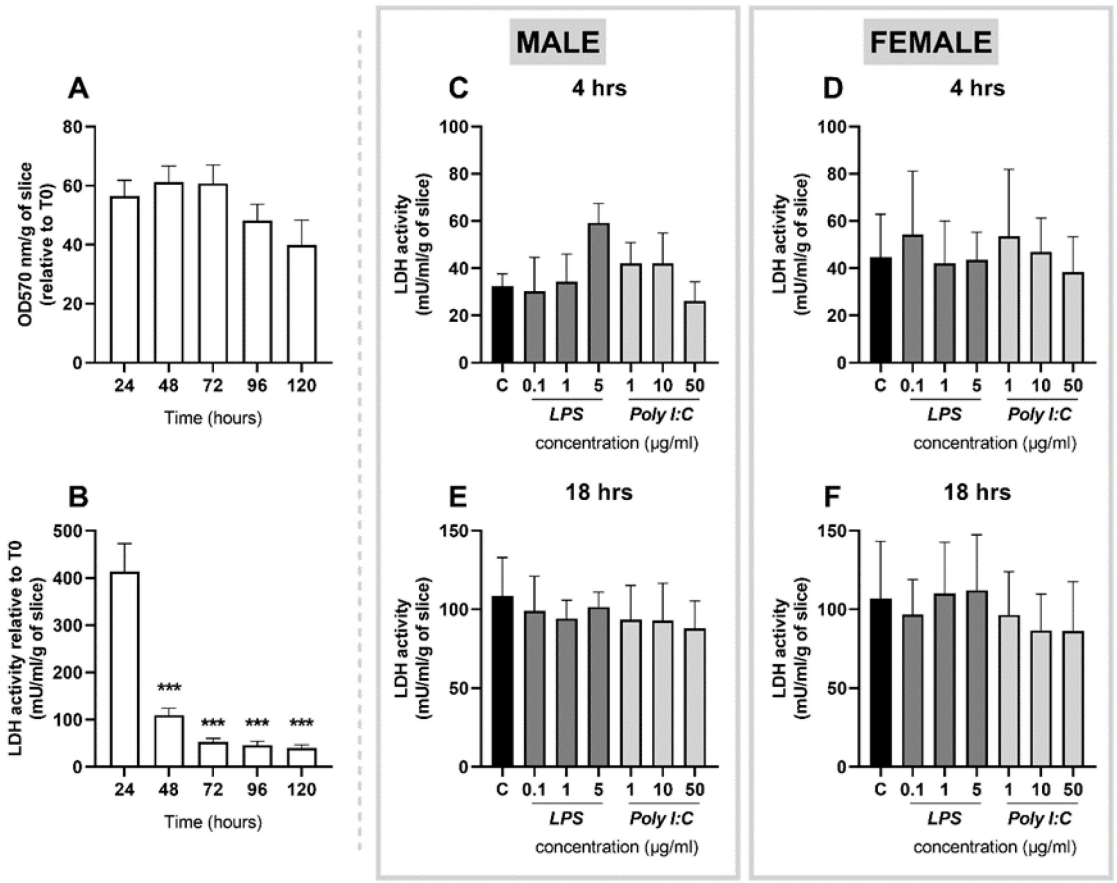

Figure 8. Assessment of viability and integrity in precision-cut rat Placental slices. (A) Viability of placental slices was determined using the MTT assay at 24-hour intervals over a 120-hour duration. (B) To evaluate tissue integrity, placenta slices were cultured for 120 hours under basal conditions, and LDH release was measured in the culture media every 24 hours. Similarly, LDH activity was assessed in male (C, E) and female (D, F) placental slices following 4 and 18 hours of LPS and Poly I:C treatment. All results were normalized to the tissue weight. All data is presented as the mean ± SEM; $n \geq 3$. *** indicates $p < 0.001$.

Effect of LPS and Poly I:C on proinflammatory cytokine response in placental slices

Exposing rat placental slices to various concentrations of LPS (0.1, 1, and 5 μg/ml) or Poly I:C (1, 10, and 50 μg/ml) for different durations (4 and 18 hours) resulted in significant changes in the expression of proinflammatory cytokines at both gene and protein levels. The treatment with LPS led to the upregulation of TNF-α gene expression in male (Figure 9A) and female (Figure 9C) placental slices after 4 hours, with the effect persisting in male placental slices at 18 hours (Figures 9B). Poly I:C treatment, on the other hand, only induced TNF-α upregulation in male placental slices after 4 hours (Figure 9 A-D).

To validate the changes in gene expression, TNF-α protein release was quantified by ELISA. Treatment with 5 μg/ml of LPS significantly increased TNF-α protein release in the culture media for both male and female placental slices, and this effect was observed at both 4 and 18 hours of treatment (Figures 9 E-H). Interestingly, the response to 18 hours of LPS treatment showed sex-dependent differences, with a 4-fold increase in TNF-α protein concentration in male placental slices (Figure 9F) and a substantial 17-fold upregulation in female placental slices compared to baseline levels (Figure 9H).

Similarly, IL-6 gene expression was significantly upregulated in male placental slices exposed to 5 μg/ml LPS at both time points (Figures 10 A&B), while female placental slices showed a weaker response (Figures 10 C&D). However, the release of IL-6 protein was significantly higher in both male and female placental slices treated with 5 μg/ml LPS for 4 and 18 hours (Figures 10 E-H). In contrast, no notable changes in IL-6 gene expression or protein concentrations were observed following Poly I:C treatment (Figures 10 A-H).

Regarding IL-1β changes were primarily observed at the gene expression level. Both male and female placental slices exhibited significantly increased IL-1β mRNA levels when treated with escalating concentrations of LPS for 4 and 18 hours (Figures 11 A-D). Poly I:C treatment also led to the upregulation of IL-1β gene expression in both male and female placental slices (Figures 11 A-C), with male placental slices showing the effect even at 18 hours of exposure (Figures 11 A&B), while female placental slices responded only at 4 hours (Figures 11C). However, the protein concentration of IL-1β was largely unaffected by either treatment, except for female placental slices exposed to a higher LPS concentration (5 μg/ml) for 4 hours (Figure 11G). Notably, under basal conditions, rat placental slices released cytokines in the following

order: IL-1β > TNF-α > IL-6 (Figures 9-11). Extended treatment durations may be necessary to observe significant effects on IL-1β protein secretion.

Overall, the findings highlight the significant upregulation of proinflammatory cytokines at both the gene and protein levels in response to LPS treatment in rat placental slices. In contrast, the response of placental slices to Poly I:C treatment in this study was generally less pronounced.

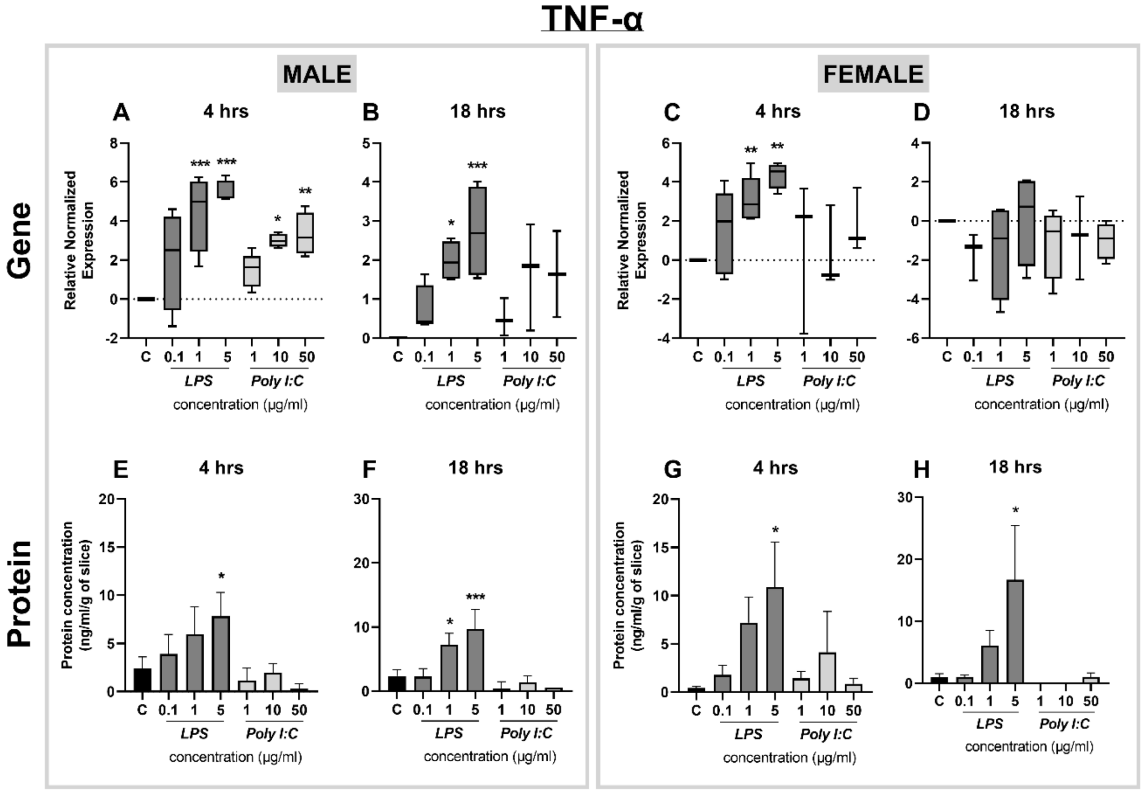

Figure 9. Assessment of the impact of LPS and Poly I:C on TNF-α gene expression and protein levels. Male and female placental slices were exposed to varying concentrations of LPS and Poly I:C for 4 and 18 hours. TNF-α gene expression in placental slices was analyzed through qPCR (A-D), while TNF-α protein concentrations in the culture media were quantified using a commercially available ELISA kit (E-H). The data is presented as Tukey boxplots or mean ± SEM; n ≥ 3. Significance levels are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

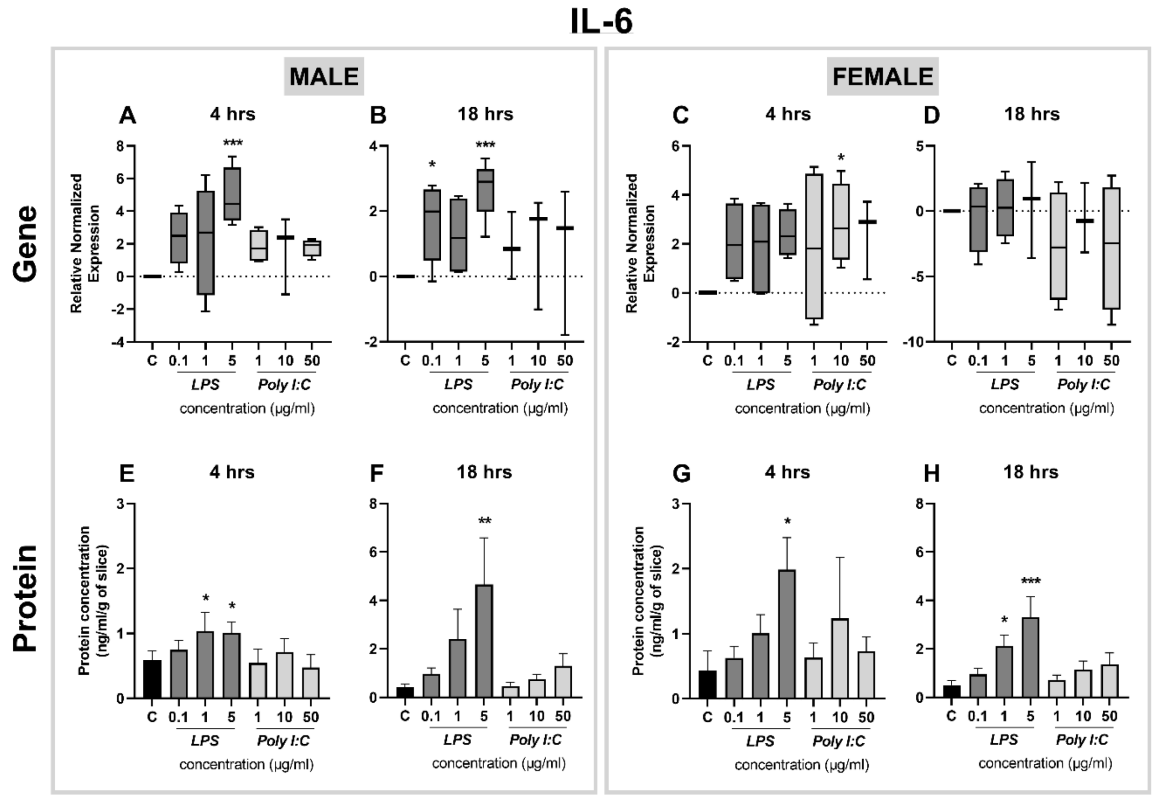

Figure 10. Impact of LPS and Poly I:C on IL-6 gene expression and protein levels. Both male and female placental slices were subjected to varying concentrations of LPS and Poly I:C for 4 and 18 hours. The gene expression of IL-6 in placental slices was quantified through qPCR analysis (A-D), while IL-6 protein concentrations in the culture media were determined using a commercially available ELISA kit (E-H). The results are presented as Tukey boxplots or mean ± SEM; $n \geq 3$. Statistical significance is denoted as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

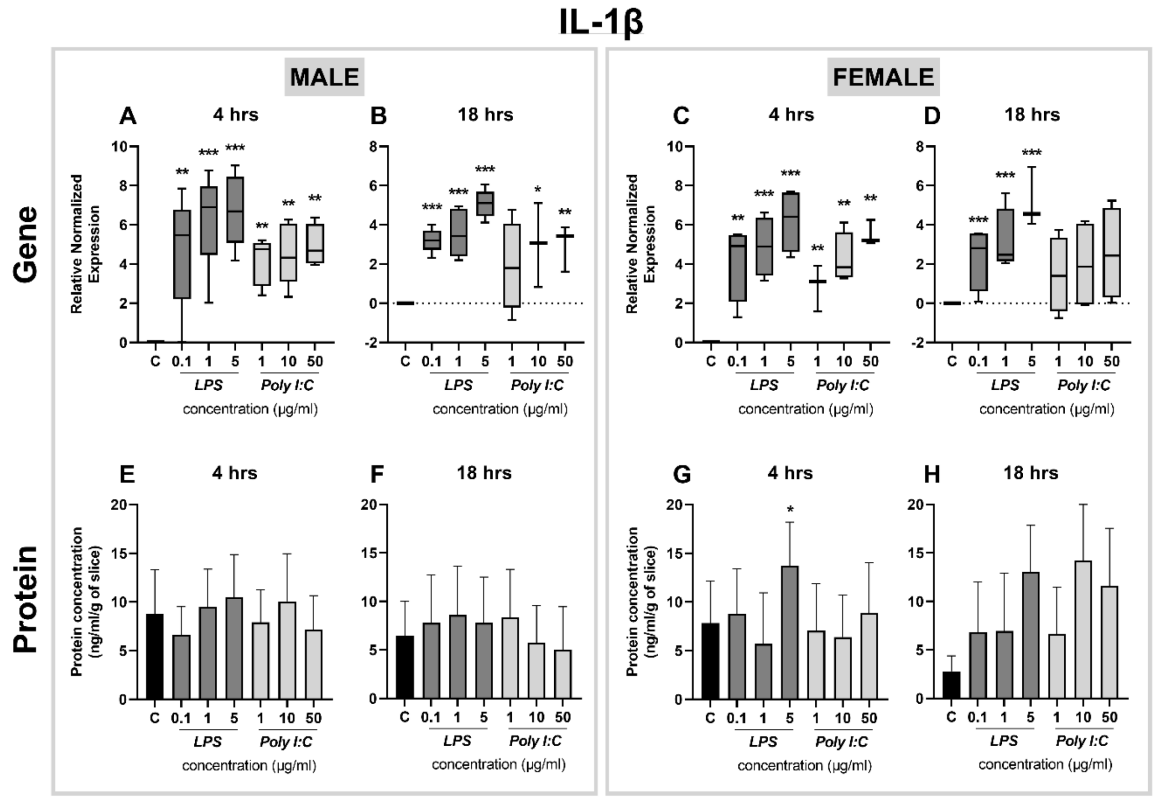

Figure 11. Impact of LPS and Poly I:C on IL-1β gene expression and protein levels. Placental slices from both male and female specimens were exposed to varying concentrations of LPS and Poly I:C for 4 and 18 hours. The gene expression of IL-1β in these slices was assessed through qPCR analysis (A-D), and IL-1β protein concentrations in the culture media were quantified using a commercially available ELISA kit (E-H). Results are depicted as Tukey boxplots or mean ± SEM; n ≥ 3. Statistical significance is represented as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

Gene expression of Tlr3 and Tlr4 in placental slices stimulated by Poly I:C and LPS

To investigate the impact of Poly I:C and LPS treatments on toll-like receptor 3 (Tlr3) and 4 (Tlr4) gene expression, we examined the expression profiles of Tlr3 and Tlr4 in rat placental slices. Tlr3 recognizes Poly I:C as its ligand, while Tlr4 recognizes LPS. Our results showed that the expression of Tlr3 gene remained unaltered after treatment with Poly I:C for both 4 and 18 hours, irrespective of the concentration (Figures 12 A-D). Similarly, stimulation of rat placental slices with LPS did not induce any significant changes in Tlr4 gene expression at any of the tested concentrations and time points (Figures 12 E-H). These findings were consistent in both male and female placentas.

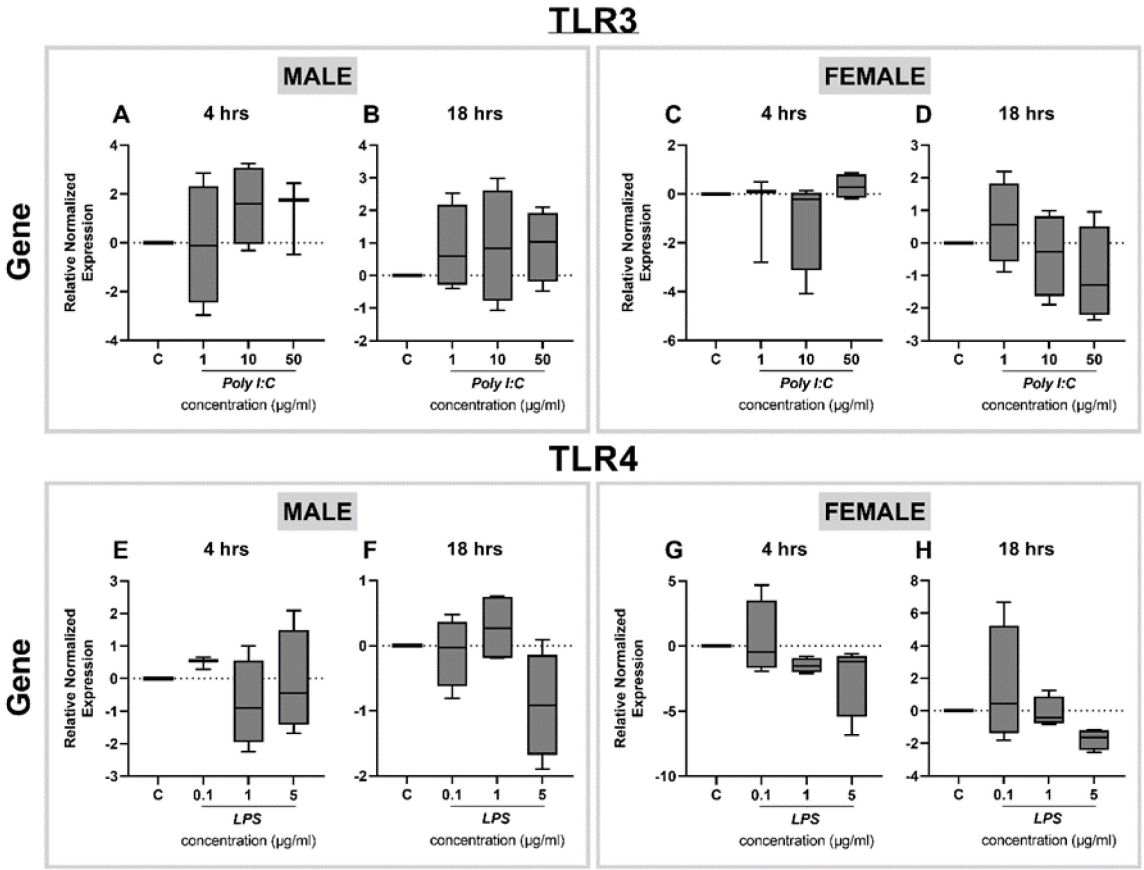

Figure 12. Impact of LPS treatment on Tlr3 and Tlr4 gene expression in rat placental slices. Both male and female placental slices were subjected to escalating concentrations of Poly I:C (A-D) and LPS (E-H) for durations of 4 and 18 hours. The qPCR analysis was employed to assess the gene expression of Tlr3 (A-D) and Tlr4 (E-H). The presented data is displayed as Tukey boxplots, with n=4 for each dataset.

7 DISCUSSION

The utilization of precision-cut slices as viable tissue explants has been extensively employed to explore metabolism, transport, and therapeutic targeting in various organs like the liver, lung, and intestinal tract (Olinga, Merema et al. 2001, Sauer, Vogel et al. 2014, Li, de Graaf et al. 2016, Liu, Särén et al. 2021). However, there has been relatively limited research on placental slices since their introduction by Gilligan *et al*., in 2012 (Gilligan, Tong et al. 2012). In this study, we present the first investigation using the precision-cut slice model to study the acute inflammatory response in the rat placenta. Our findings demonstrate the activation of proinflammatory cytokines in placental slices upon exposure to the Tlr4 ligand, LPS, in a sex-dependent manner.

Cell viability is crucial in tissue culture models, and in line with Gilligan et al., our results indicate that LDH release, a marker of tissue injury, peaks during the initial 24 hours of culture. Subsequently, LDH release significantly decreases after 48 hours, remain stable up to 120 hours. Similarly, the MTT assay, which assesses cellular metabolic activity, reveals no significant reduction in placental slice viability for up to 120 hours. These observations are consistent with prior studies (Gilligan, Tong et al. 2012, Sauer, Vogel et al. 2014, Wu, Roberto et al. 2018) , These results demonstrate that tissue integrity is compromised within the first 24 hours following the slicing procedure, necessitating a recovery period before initiating experiments. Consequently, all treatment studies in this research were conducted 36 hours after slice preparation. To verify that LPS and Poly I:C do not exert cytotoxic effects on rat placental slices, LDH release was evaluated after 4 and 18 hours of treatment. Our results confirm that LPS and Poly I:C do not cause tissue damage in placental slices under the tested concentrations and exposure times used in our study. Therefore, any observed tissue response in the subsequent experiments is solely attributable to the proinflammatory processes induced by LPS and Poly I:C.

Inflammation during pregnancy is increasingly recognized to create a hostile proinflammatory environment in the fetoplacental unit, characterized by an upsurge in cytokine release (Ratnayake, Quinn et al. 2013, Zhang, Luo et al. 2018). A preclinical placental inflammatory model serves as a potent tool to unravel the tissue response to immune-activating agents and explore the downstream effects of the proinflammatory cytokine network. In previous studies using other placental models, elevated levels of proinflammatory cytokines, particularly TNF-α, IL-6, and IL-1β, have been reported following treatment with LPS and Poly I:C (Boles, Ross

et al. 2012, Bloise, Bhuiyan et al. 2013, Lye, Bloise et al. 2015, McColl and Piquette-Miller 2019) . However, the application of precision-cut slices to investigate inflammatory responses has predominantly been tested in liver and lung slices from piglets and mice (Liu, Särén et al. 2021, Hasuda, Person et al. 2022). In this study, we demonstrate that exposing rat placental slices to LPS significantly increases the expression and secretion of key proinflammatory cytokines, namely TNF-α, IL-6, and IL-1β. This indicates the activation of the Tlr4-dependent NF-κB pathway, leading to the induction of inflammatory genes (Boles, Ross et al. 2012, Fan, Li et al. 2019).

Regarding Tlr4 expression, previous investigations have yielded conflicting findings. While some studies have shown no effect on Tlr4 expression upon LPS treatment (Boles, Ross et al. 2012, Bloise, Bhuiyan et al. 2013, Lye, Bloise et al. 2015), in contrast others have reported upregulation of placental Tlr4 expression upon LPS stimulation (Xue, Zheng et al. 2015, Fan, Li et al. 2019) . In our study, we observed that Tlr4 gene expression remained unaltered regardless of the LPS concentration, time of exposure, or fetal sex. Thus, the induction of cytokines in placental slices is likely attributed to the activation of the Tlr4 downstream signaling pathway.

Conversely, Poly I:C stimulation resulted in a less pronounced inflammatory response; however, it led considerable expression of proinflammatory cytokine gene in both male and female placentas. As Poly I:C targets Tlr3, we investigated whether the observed hypo-responsiveness to Poly I:C could be attributed to the regulation of Tlr3 gene expression in rat placental slices. Nevertheless, we detected no changes in Tlr3 gene expression regardless of the dose, exposure time, or fetal sex. Considering that the placenta demonstrates a heightened response to infection in earlier stages of pregnancy compared to term (Mor and Cardenas 2010), we speculate that the gestational age chosen for our studies (term placenta) may influence the magnitude of the immune response. Additionally, potential endotoxin contamination and molecular weight variations in the Poly I:C composition have been reported to introduce variability in immune responses in vitro and in vivo in rats (Zhou, Guo et al. 2013, Kowash, Potter et al. 2019), which could have influenced our Poly I:C experimental results and necessitate further investigation.

The rat placental slice model offers a significant advantage in studying sex-dependent processes as it allows the generation of slices from both male and female placentas obtained from the same dam. Nevertheless, conflicting reports on sexual dimorphism in the placental

inflammatory response have been published. Some recent studies have indicated that the male placenta exhibits a more robust response to LPS, characterized by higher levels of proinflammatory cytokines TNF-α and IL-6 (Cvitic, Longtine et al. 2013, Cai, van Mil et al. 2016, Everhardt Queen, Moerdyk-Schauwecker et al. 2016, Braun, Carpentier et al. 2019). Conversely, others have demonstrated that the female placenta shows a stronger proinflammatory response to various inflammatory stimuli (Verthelyi 2001, Sood, Zehnder et al. 2006, Di Renzo, Picchiassi et al. 2015). In our study, we observed that placental slices prepared from female placentas exhibited higher sensitivity to LPS, leading to a larger secretion of proinflammatory cytokines, particularly TNF-α.

This disparity in response could be attributed to the level of sex hormones, such as estrogens and testosterones, produced by the placenta. Estrogen, known to induce NF-κB signaling, acts as an immune enhancer, while testosterone generally functions as an immune suppressor (Di Renzo, Picchiassi et al. 2015, Cai, van Mil et al. 2016). Additionally, several studies have reported higher expression levels of cytokine receptors in female placentas compared to male placentas (Sood, Zehnder et al. 2006, Di Renzo, Picchiassi et al. 2015, Cai, van Mil et al. 2016). Nonetheless, to fully understand the molecular mechanisms behind placental sexual dimorphism, further investigations are warranted.

Innate immune responses at the decidual-placental interface are orchestrated by a diverse range of maternal and fetal cells, contributing to the dynamic interplay that regulates placental immune functions (Hoo, Nakimuli et al. 2020). The rat placental slice model, by preserving the multicellular architecture, cell-cell interactions, and three-dimensional structure of the tissue, provides a valuable platform to investigate the intricate immunological processes that occur within this vital interface. Moreover, this model allows the use of placentas from diseased pregnancies to study pathological processes and investigate potential therapeutic interventions. By adhering to the 3R principles, the high-throughput nature of this model can significantly reduce the number of animals used for experimentation, while also minimizing discomfort to the animals due to the ex vivo nature of the manipulations (Li, de Graaf et al. 2016).

However, it is important to acknowledge the limitations of this model. Careful consideration of species differences is essential to ascertain the translatability of findings to humans. While rat placental slices provide valuable insights, it is crucial to determine the model's applicability to specific biological processes accurately. Additionally, the relatively short culture duration

of up to 4 days may limit the evaluation of chronic xenobiotics exposure, prompting researchers to adopt other suitable models for such studies.

In summary, the rat placental slice model stands as a powerful and versatile tool in immunological research, providing an ex vivo platform that preserves tissue architecture and function while adhering to ethical research principles. By understanding its strengths and limitations, researchers can harness the potential of this model to gain valuable insights into placental immune responses and further our understanding of pregnancy-related pathologies and potential therapeutic interventions.

8 CONCLUSION

In conclusion, our findings highlight the valuable applicability of rat placental precision-cut slices as an effective experimental tool for the investigation of inflammatory processes induced by both bacterial (LPS) and viral (Poly I:C) agents. Additionally, this model provides a platform to examine Tlr4 activation and explore the impact of sexual dimorphism on immune responses. Given the pivotal role of maternal inflammation in influencing pregnancy outcomes and fetal well-being, this experimental approach offers a promising avenue to unravel the intricate placental pathways involved in fetal programming. Furthermore, its potential application spans across diverse fields, encompassing immunology, pharmacology, and toxicology, where it holds promise for advancing our understanding and addressing the implications of inflammation during pregnancy.

The data presented in this study have been published in *Frontiers in Immunology* (Anandam, Abad et al. 2022); the student is a co-author of the paper.

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