Université de Montréal

Born Too Small, Too Soon: How Can We Save Them?

A Novel Interleukin-1 Antagonist, Rytvela, Successfully Reverses the Inflammatory Cascade Leading to Intrauterine Growth Restriction and Preterm Birth

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Ce mémoire intitulé

A Novel Interleukin-1 Antagonist, Rytvela, Successfully Reverses the Inflammatory Cascade Leading to Intrauterine Growth Restriction and Preterm Birth

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Résumé

Contexte : Près de 2,5 millions de nouveau-nés meurent chaque année et plus de 80 % d'entre eux ont un petit poids à la naissance (PPN). Le PPN est une entité clinique complexe impliquant le retard de croissance *in utero* (RCIU) et la naissance prématurée (NPM). Les nouveau-nés survivants sont exposés à un risque élevé de morbidités périnatales graves (telles que la dysplasie broncho-pulmonaire, l'entérocolite nécrosante, l'encéphalopathie néonatale) en raison des effets dévastateurs de l'inflammation utéro-fœtale sur les organes fœtaux vulnérables. Il n'existe actuellement aucun traitement efficace pour la protection fœtale ante partum. Parmi les nombreux médiateurs pro-inflammatoires, l'IL-1β se distingue par ses effets délétères. Notre laboratoire a conçu un nouvel antagoniste allostérique du récepteur de l'IL-1, Rytvela, qui s'est avéré efficace contre la NPM lorsqu'il est administré en prophylaxie. **Objectif** : Cette étude vise à mieux caractériser Rytvela en évaluant son efficacité dans la prévention de la NPM et du RCIU lorsqu'il est administré après l'insulte inflammatoire initiale selon un cadre clinique plus réaliste. **Méthodes** : Des souris gravides CD-1 ont reçu une injection d'agents pro-inflammatoires/pro-travail, soit l'IL-1β (1 µg i.u.) ou le LPS (10 µg i.p.) aux jours 16-17 de la gestation. Rytvela (2 mg/kg/jour s.c.) a été administré à différents intervalles de temps (0,5h, 2h, 4h, 6h) après l'induction inflammatoire. Le taux de NPM, la survie et le poids des souriceaux ont été évalués. Des analyses histologiques des poumons, intestins et cerveau des nouveau-nés ont été réalisées. **Résultats** : Toutes les grossesses traitées avec Rytvela ont été menées à terme dans le modèle de l'IL-1β, alors que le taux de NPM était de 57 % dans le groupe non traité. La survie, la croissance et le poids des souriceaux ont été considérablement améliorés avec Rytvela administré 0,5 h post-inflammation (avec une survie presque doublée des portées). L'analyse histologique a révélé dans tous les modèles une morphogenèse fœtale protégée, y compris une alvéolarisation pulmonaire préservée, des villosités intestinales intactes et un arbre cérébrovasculaire protégé associé à une masse cérébrale préservée. **Conclusion** : Rytvela est efficace dans la prévention de la NPM et du RCIU lorsqu'il est administré en post-inflammatoire. Il présente un effet maximal lorsqu'il était administré rapidement (0,5 h après IL-1β/LPS) et maintenait des effets protecteurs fœtaux significatifs avec une administration retardée (jusqu'à 6 h après IL-1β/LPS). Rytvela améliore la

survie et la santé néonatale en préservant l'intégrité et la croissance des tissus fœtaux. Par conséquent, Rytvela est un nouveau prototype thérapeutique prometteur et sécuritaire pour le traitement de la NPM et du RCIU.

Mots-clés : restriction de croissance *in utero*, retard de croissance fœtal, naissance prématurée, travail prématuré, Rytvela, inflammation, interleukine-1, dysplasie bronchopulmonaire, entérocolite nécrosante, encéphalopathie néonatale.

Abstract

Background: Over 2.5 million newborns die yearly and more than 80% of them are of low birthweight (LBW). LBW is a complex clinical entity involving fetal growth restriction (FGR) and preterm birth (PTB). Surviving neonates face a higher risk of serious perinatal morbidities (such as bronchopulmonary dysplasia, necrotizing enterocolitis, neonatal encephalopathy) due to the devastating effects of utero-fetal inflammation on vulnerable fetal organs. There is currently no efficient treatment for fetal antepartum protection. Among the many proinflammatory mediators, IL-1β stands out for its detrimental effects. The host lab has designed a novel allosteric IL-1 receptor antagonist, Rytvela, which has been shown to be effective against PTB when administered prophylactically. **Objective**: This study aims to further characterize Rytvela by evaluating its efficacy in preventing PTB and FGR when administered after the initial inflammatory insult according to a more realistic clinical setting. **Methods**: Pregnant CD-1 mice were injected with proinflammatory/prolabour agents, either IL-1β (1 µg i.u.) or LPS (10 µg i.p.) on days 16-17 of gestation. Rytvela (2 mg/kg/day s.c.) was administered at different time intervals (0.5, 2, 4, 6 h) after initial inflammatory insults. PTB rate, neonatal survival, and weight were assessed. Histological analyses of the lungs, intestines, and brain of the neonates were performed. **Results**: All pregnancies treated with Rytvela were carried to term in the IL-1β model, while the PTB rate was 57% in the untreated group. Pup survival, growth and weight were considerably improved with Rytvela administered 0.5h post-inflammatory insults (with a nearly 2-fold increase in litters survival). Histological analysis revealed in all models a protected morphogenesis of vulnerable fetal organs including preserved lung alveolarization, intact intestinal villi integrity, and protected cerebrovascular tree associated with preserved brain mass. **Conclusion**: Rytvela is efficient in preventing PTB and FGR when administered post-inflammatory insults. It exhibited maximum effect when administered promptly (0.5h post-IL-1β/LPS) and maintained significant fetal protective effects with delayed administration (up to 6h post- IL-1β/LPS). Rytvela improved birth outcome by preserving fetal tissue integrity and growth. Hence, Rytvela is a promising new and safe therapeutic prototype for treatment of PTB and FGR.

Keywords: Intrauterine growth restriction, Fetal growth restriction, Preterm birth, Preterm labour, Rytvela, Inflammation, Interleukin-1, Bronchopulmonary dysplasia, Necrotizing enterocolitis, Neonatal encephalopathy.

Table of Content

List of Tables

List of Figures

Figure 23 Microvascular degeneration in brain induced by antenatal two-time exposure to LPS is prevented with the administration of Rytvela after inflammatory onset. (**A**) Representative image of cortex vasculature and quantification of vessel density. Immunostaining for lectin (vasculature) is shown in fluorescent green. Vascular density measurements were performed on 5-25 region of interest (ROI) per group using ImageJ software. Scale of images: 750 X 750 µm. (**B**) Region quantified is represented on the full cerebral coronal section, stained with DAPI in blue. ROI of 750 X 750 µm is shown in yellow (**C**) Brain weight of pups on post-term day 7 (PT7). Brains were all collected on 2 or more pups per litter at PT7, from 3-7 dams per group. *n* = 7-18 pups per group for A-B, and 4-10 pups per group for C. Values are presented as mean ± SEM. ***p* < 0.01, *****p* < 0.0001 by one-way ANOVA with Dunnett's test. 100

List of Acronyms and Abbreviations

A: Alanine

ACOG: American College of Obstetricians and Gynecologists

AI: Artificial Intelligence

AMA: Advanced Maternal Age

ANOVA: Analysis of Variance

AP-1: Activator Protein 1

ASA: Acetylsalicylic Acid

BPD: Bronchopulmonary Dysplasia

CCB: Calcium Channel Blocker

CCL2: CC-chemokine Ligand 2

CCL4: CC-chemokine Ligand 4

CHUSJ: Centre Hospitalier Universitaire Sainte-Justine

CLD: Chronic Lung Disease

CNS: Central Nervous System

COX-1: Cyclooxygenase 1

COX-2: Cyclooxygenase 2

CP: Cerebral Palsy

CXCL1: C-X-C Motif Chemokine Ligand 1

CX-43: Connexin 43

DAG: Diacyl Glycerol

DAMPs: Damage-Associated Molecular Patterns

DAPI: 4’,6-diamidino-2-phenylindole

DNA: Deoxyribonucleic Acid

E: Glutamate

EGF: Epidermal Growth Factor

ELISA: Enzyme-Linked Immunosorbent Assay

ET-1: Endothelin-1

FDA: Food and Drug Administration

FiO2: Fraction of inspired Oxygen

FIRS: Fetal Inflammatory Response Syndrome

FITC: Fluorescein Isothiocyanate

FGR: Fetal Growth Restriction

FSC: Frozen Section Compound

G11: Gestational day 11

G16: Gestational day 16

G16.5: Gestational day 16.5

G17: Gestational day 17

G18.5: Gestational day 18.5

G19: Gestational day 19

GA: Gestational Age

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

H&E: Hematoxylin and Eosin

HMD: Hyaline Membrane Disease

HMGB1: High–Mobility Group Box 1

HPS: Hematoxylin/Phloxine/Safran

IκB: IkappaB kinase

IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IKKs: IkappaB kinases

IL-1: Interleukin-1

IL-1β: interleukin-1β

IL-1R: Interleukin-1 Receptor

IL-1RI: Interleukine-1 Receptor subunit I

IL-1Ra: Interleukin-1 Receptor antagonist

IL-1RacP: Interleukine-1 Receptor accessory Protein

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-10: Interleukin-10

IL-11: Interleukin-11

IL-15: Interleukin-15

IL-18: Interleukin-18

IL-33: Interleukin-33

iNOS: inducible Nitric Oxide Synthase

i.p.: intraperitoneally

IP3: Inositol trisphosphate

i.u.: intrauterine

IUGR: Intrauterine growth restriction

IV: Intravenous

IVH: Intraventricular Hemorrhage

JNK: c-Jun N-terminal Kinases

L: Leucine

LBW: Low Birth Weight

LIF: Leukemia Inhibitory Factor

LPS: Lipopolysaccharide

LTA: Lipoteichoic Acid

MAPK: Mitogen-Activated Protein Kinases

MRHD: Maximum Recommended Human Dose

MgSO4: Magnesium Sulfate

MNICU: Mouse Neonatal Intensive Care Unit

MMPs: Matrix Metalloproteinases

MMP9: Matrix Metalloproteinase 9

MRI: Magnetic Resonance Imaging

mRNA: messenger Ribonucleic Acid

NBW: Normal Birth Weight

NE: Neonatal Encephalopathy

NEC: Necrotizing Enterocolitis

NF-kB: Nuclear Factor kappa-B

NICU: Neonatal Intensive Care Unit

NK cells: Natural Killer cells

NO: Nitric Oxide

NPM: Naissance Prématurée

NSAID: Nonsteroidal Anti-Inflammatory Drug

NTB: Normal Term Birth

OXTR: Oxytocin Receptor

P4: Progesterone

PAMPs: Pathogen-Associated Molecular Patterns

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDA: Patent Ductus Arteriosus

PGE2: Prostaglandine E2

PLV: Periventricular Leukomalacia

PO: Per Os

PPN: Petit Poids à la Naissance

PPROM: Preterm Premature Rupture of Membranes

PROM: Premature Rupture of Membranes

PRRs: Pattern Recognition Receptors

PT: Post-Term day

PT7: Post-Term day 7

PT15: Post-Term day 15

PTB: Preterm Birth

PTGS2: Prostaglandin-Endoperoxide Synthase 2

PTL: Preterm Labor

PVL: Periventricular Leukomalacia

qPCR: quantitative Polymerase Chain Reaction

R: Arginine

RCIU: Retard de Croissance In Utero

RDS: Respiratory Distress Syndrome

ROCK: Rho-associated Coiled-coil-containing Kinase

ROI: Region of Interest

ROP: Retinopathy of Prematurity

ROS: Reactive Oxygen Species

SAPK: Stress-Associated Protein Kinase

s.c.: subcutaneous

SD: Standard Deviations

SEM: Standard Error of the Mean

SGA: Small for Gestational Age

SOGC: Society of Obstetricians and Gynaecologists of Canada

T: Threonine

TGF-α: Transforming Growth Factor alpha

TGF-β: Transforming Growth Factor beta

TGF-β2: Transforming Growth Factor beta 2

Th1: T-helper 1 lymphocyte

Th2: T-helper 2 lymphocyte

TLR: Toll-Like Receptor

TLR4: Toll-Like Receptor 4

TLR9: Toll-Like Receptor 9

TNF: Tumor Necrosis Factor

TNFα: Tumor Necrosis Factor-alpha

TORCH screen: Toxoplasmosis, Other, Rubella, Cytomegalovirus, Herpes simplex virus screen.

TTTS: Twin-to-Twin Transfusion Syndrome

TXA2: Thromboxane A2

UAP: Uterine Activation Proteins

V: Valine

VEGF: Vascular Endothelial Growth Factor

Y: Tyrosine

Dedicated to Dr. Sylvain Chemtob,
for his trust, support, and encouragement
from the very first day to the very last.

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INTRODUCTION

1. Intrauterine Growth Restriction (IUGR) and Preterm Birth (PTB)

1.1 History and Definitions

Historically, a baby was deemed to be born prematurely if it weighted less than 2500 g at birth. For instance, the World Health Organization's original definition of prematurity was based on low birth weight (LBW). In the 1950s, focus turned to two etiologies of LBW, preterm births (PTB) and intrauterine growth restriction (IUGR), which were both causally associated with morbidity and mortality. Studies shifted from using birth weight to using gestational age to define and establish prematurity, and eventually estimated fetal weight by performing ultrasound scans during pregnancy to correlate the evolution of fetus weight to a mean for gestational age. (1)

PTB is now defined by the World Health Organization as birth occurring before 37 completed weeks of gestation (2). Normal term pregnancy duration is deemed to be 37 to 42 weeks. (3) We can further subdivide prematurity into: moderate to late preterm (32 to 37 weeks), very preterm (28 to 32 weeks), extremely preterm (less than 28 weeks). (2, 3, 4)

IUGR, on the other hand, refers to babies that are unable to reach their full growth potential *in utero*. In clinical practice, it is difficult to ascertain the growth potential of a fetus. The most widely accepted definition of IUGR refers to babies that are small for their gestational age (SGA), i.e., babies whose weights is either below the 10^{th} percentile for all babies of the same gestational age, or less than two standard deviations (SD) from the mean for gestational age. The difference between IUGR and SGA is that IUGR babies saw their growth restricted *in utero* due to maternal, fetal, or placental pathology, whereas SGA babies are just constitutionally small, without any known underlying pathological cause. The generally accepted clinical definition of IUGR is not comprehensive. Some babies are born with a weight greater than the 10^{th} percentile but still present an IUGR for example a newborn weighting at the 25^{th} percentile while his potential is at the 75^{th} percentile. This baby could fall outside of this clinical IUGR definition. (4, 5, 6)

Despite the above specificities of prematurity, the World Health Organization still uses LBW (birth weight lower than 2500 g) as a blanket criterion for statistical data. It identifies the majority of newborns requiring special attention, mainly due to either prematurity or IUGR. (4, 7)

Table 1 is intended to serve as a reference for important terminology and definitions discussed in this section.

Intra-Uterine Growth Restriction (**IUGR**)	Full growth potential not reached *in utero* due to underlying pathology. Clinically diagnosed using SGA scales.
Fetal Growth Restriction (**FGR**)	Recent synonym of IUGR.
Small for Gestational Age (**SGA**)	Weight < 10th percentile, or < 2 SD from the mean weight for GA, without underlying pathological cause.
Low Birth Weight (**LBW**)	< 2500 g
Very **LBW**	< 1500 g
Extremely **LBW**	< 1000 g
Normal Birth Weight (**NBW**)	2500-4499 g
Pre-Term Birth (**PTB**)	< 37 weeks of gestation
Moderate to Late **PTB**	32-37 weeks of gestation
Very **PTB**	28-32 weeks of gestation
Extremely **PTB**	< 28 weeks of gestation
Normal Term Birth (**NTB**)	37-42 weeks

Tableau 1 Summary of important terminology related to perinatology

1.2 Statistics

Each year, an estimated 15 million newborns are born preterm and more than 20 million are born with low birth weight. (2, 7, 8) Intrauterine growth restriction and preterm birth account for a large share of global child mortality and morbidity. Prematurity and low birth weight remains the leading cause of death in newborns and children under-five years worldwide. (8, 9) Newborn mortality accounts for nearly 2.5 million deaths yearly and more than 80% of these newborns

suffer from low birthweight. (7) PTB is rising rapidly around the world : incidence ranges from 5% to 18% of births. (9) In the United States, more than 1 in 10 babies are born preterm each year. PTB rates reached 10.5% in 2021. (10) In Canada, PTB and SGA rates are approximately 8.1% and 8.3%, respectively, accounting for more than 25,000 live births combined yearly. (11, 12) PTB accounts for nearly two thirds of infant deaths in Canada. (5) The surviving preterm and growth restricted newborns are predisposed to a higher risk of perinatal morbidities (such as respiratory distress syndrome, bronchopulmonary dysplasia, necrotizing enterocolitis, retinopathy of prematurity, cerebral palsy, and other neurodevelopmental deficits, that will be discussed in the below sections). Preterm babies often stay in the Neonatal Intensive Care Unit (NICU) for a prolonged period. Growth restricted infants are more likely to remain in neonatal intensive care unit (NICU) significantly longer than gestation age-matched infants. (13) These babies account for a disproportionately high percentage of healthcare costs among newborns. (14) PTB is estimated to cost the Canadian health care system over $8 billion per year. (14) The annual cost in the United States is at least $26.2 billion per year and climbing. (15) PTB and LBW may have consequences that continue into adulthood, increasing the risk of adult-onset chronic conditions and lifetime disabilities such as cardiovascular disease, obstructive pulmonary disease, renal impairment, obesity, diabetes, neurodevelopmental disorders, such as cerebral palsy, intellectual disabilities, and vision/hearing impairments. (8, 13, 16) These chronic medical and neurological complications often require additional healthcare and educational services, which add to the overall economic cost of caring for these fragile infant over their lifetimes. (17)

1.3 Etiologies and Risk Factors

The exact etiologies of IUGR and PTB are currently mostly unknown. However, certain risk factors have been linked to the development of these conditions. We can classify these risk factors into three broad categories: maternal, placental / uterine, and fetal. These risk factors are listed in Table 2. (3, 4, 5, 15, 18, 19) Most risk factors can predispose babies to both IUGR and PTB, but some risk factors are more specific to only one of these perinatal outcomes. Interestingly, IUGR is also a risk factor for PTB.

	IUGR	PTB
Maternal	Age (< 17 or > 35) Malnutrition Substance abuse (alcohol, smoking, recreational drugs) Psychosocial stressors Pre-eclampsia Chronic diseases (hypertension, diabetes, anemia, chronic renal disease, pulmonary and cardiovascular diseases, systemic autoimmune diseases) Rh isoimmunization Infections (urinary tract infections, bacterial vaginosis, trichomoniasis, sexually transmitted infections, chorioamnionitis, HIV, Group B strep, toxoplasmosis, syphilis, varicella-zoster, parvovirus B19, rubella, cytomegalovirus, herpes, malaria, tuberculosis) History of previous IUGR / PTB	
	Hypoxia (high altitude) Drugs (warfarin, steroids, anticonvulsants, antineoplastic)	
Placental / Uterine	Placental anomalies (abnormal placental vasculature, placental infarction, placental abruption, placental insufficiency, placenta previa, abnormal insertion of the umbilical cord, single umbilical artery) Uterine malformations (bicornuate uterus, uterine fibroids) Multiple gestation (twins, triplets, or more) Partial molar pregnancy Twin-to-twin transfusion syndrome (TTTS) premature rupture of membranes (PROM)	
		Uterine overdistension (polyhydramnios, macrosomia) Intrauterine bleeding (antepartum hemorrhage) Placental senescence Cervical insufficiency
Fetal	Congenital anomalies (tracheoesophageal fistulas, congenital heart defects, anencephaly, gastroschisis, central nervous system defects, orofacial or musculoskeletal defects) Chromosomal abnormalities (Trisomy 21, 18, 13, Turner syndrome)	
	Genetic syndromes (Bloom, Seckel, Russell-Silver syndrome) Metabolic disorders (galactosemia, phenylketonuria)	Intrauterine growth restriction (IUGR). Fetal distress (spontaneous or iatrogenic)

Tableau 2 Common and distinct risk factors for IUGR and PTB

In short, PTB and IUGR are not seen as a single disease entity but are referred to as syndromes attributable to multiple pathological processes. A significant proportion of these broad etiologic factors have a common characteristic in their pathophysiology: they lead to detrimental fetomaternal inflammation. The central role of inflammation in PTB and IUGR will be further described in section 2 of the introduction.

1.4 Frequent Complications of IUGR and PTB

IUGR and PTB syndromes not only have common causes, they also have several common consequences. The severity of these complications generally increases in an inversely proportional manner to the gestational age and birth weight of the newborn. In other words, the risk of serious complications is obviously higher if the baby presents a more severe IUGR and/or if the baby is born further from his or her expected term.

Infants with IUGR are more frequently prone to the following short-term complications after birth: neonatal asphyxia, meconium aspiration, persistence of fetal circulation, patent ductus arteriosus, persistent pulmonary hypertension, pulmonary haemorrhage, bronchopulmonary dysplasia (BPD), blood hyperviscosity or polycythaemia, leukoneutropenia and thrombocytopenia, immunodeficiency, jaundice, hypothermia, hypocalcaemia, hypoglycemia, feed intolerance, necrotizing enterocolitis (NEC), late-onset sepsis, renal tubular injury, intraventricular hemorrhage, periventricular leukomalacia, retinopathy of prematurity (ROP). (4, 13, 20)

Similarly, infants with PTB are more frequently prone to respiratory, cardiovascular, gastrointestinal, neurologic, ophthalmologic, metabolic, and hematologic short-term complications after birth. These common complications include: apnea of prematurity, respiratory distress syndrome (RDS) or also called hyaline membrane disease (HMD), bronchopulmonary dysplasia (BPD), patent ductus arteriosus, feeding difficulties, necrotizing enterocolitis (NEC), intraventricular hemorrhage (IVH), periventricular leukomalacia (PLV), cerebral palsy, retinopathy of prematurity (ROP), hypothermia, hypoglycemia, anemia, unconjugated hyperbilirubinemia and jaundice, infections and sepsis. (21, 22, 23, 24)

Vulnerability is primarily observed in organs that are particularly sensible to inflammatory stressors at an early stage of development like the lungs, intestines, and brain. (25) Indeed, RDS, BPD, NEC, IVH, PVL, cerebral palsy, in both growth restricted and preterm neonates, are serious neonatal sequelae with significant lifelong consequences. (26) They will be discussed in further details in the next sections.

1.4.1 Respiratory Distress Syndrome / Hyaline Membrane Disease

Respiratory distress syndrome (RDS) of newborn, also known as hyaline membrane disease (HMD), is classically associated with prematurity. IUGR was also identified as a risk factor. (27) It is the primary cause of death in the NICU. (27) It typically worsens over the first 48 to 72 hours of life. It is mainly caused by surfactant deficiency. Surfactant is made by the type II alveolar epithelial cells and consists of phospholipids and proteins. (28) It begins to be produced in the healthy fetus at about 24 to 28 weeks of pregnancy and is usually found in amniotic fluid between 28 and 32 weeks. By about 35 weeks gestation, most babies have developed adequate amounts of surfactant. (29) When there is not enough surfactant, the lungs are unable to be expanded with air (poor lung compliance due to high alveolar surface tension) and collapse during expiration, leading to atelectasis. An inflammatory response is triggered in the lungs by a combination of different mechanisms both antepartum (disadvantageous intrauterine environment ex. chorioamnionitis or preeclampsia) and postpartum (ex. atelectasis, mechanical ventilation, hyperoxia due to supplemental oxygen). (30, 31) Chemotactic activity and inflammatory products, such as the proinflammatory cytokines IL-1, IL-6, TNF-α, IL-11, VEGF, TGF-α and TGF-β, provoke serious damage to the capillary endothelium and the alveolar epithelium, resulting in hyaline membrane formation and leakage of edema into the alveoli. (32, 33) The combination of atelectasis, accumulation of hyaline membranes, and edema results in poor surface area for gas exchange, hypoxia, acidosis and respiratory distress. (3)

Existing prevention therapy for HMD consist in maternal administration of a single or repeated intramuscular injection of betamethasone or dexamethasone (e.g. Celestone® 12 mg q24 h x 2 doses) within a time window of 24 h to 7 days prior to birth to stimulate surfactant production and induce fetal lung maturation. Indeed, the natural increased fetal production of glucocorticoids prior to normal birth has a major role in remodeling the lung extracellular matrix, reducing interalveolar tissue volume, stimulating surfactant production, and enhancing the mechanical properties of the lung. (29) Nevertheless, the best way of preventing HMD remains by preventing PTB and IUGR. More efforts should be devoted to antenatal prevention of the disease. (33) Postnatal treatment for HMD include supplemental oxygen, ventilation, and artificial surfactant. (3, 32) However, prolonged ventilation increases risk of bronchopulmonary dysplasia.

(32) Indeed, a frequent complication of HMD is chronic lung disease (CLD), also called bronchopulmonary dysplasia (BPD), which will be discussed in the next section.

It is interesting to note that surfactant proteins are also important in host defense. (29) Babies with IUGR and PTB may be at increased risk for respiratory infection.

1.4.2 Bronchopulmonary Dysplasia / Chronic Lung Disease

As discussed in the section above, growth restricted and premature babies' lungs are fragile. BPD, also called CLD, is one of the most common adverse outcome in the preterm neonates. (34) Intrauterine growth restriction was also shown to independently increases the risk of BPD, and prolongs the duration of mechanical ventilation. (35) The peak incidence of BPD is towards more immature children (less than 1250 g or 28 weeks of gestational age). (4) Diagnosis is based on prolonged (≥ 28 days or ≥ 36 weeks postmenstrual age) need for oxygen supplementation and/or ventilatory support. (3, 34)

BPD is a disease characterized by disrupted alveolar development of the immature lung, secondary to perinatal inflammatory phenomena. BPD has a highly multifactorial nature. As mentioned before, both prenatal (disadvantageous intrauterine environment e.g., infections like chorioamnionitis, preeclampsia, smoking) and postnatal (e.g., invasive mechanical ventilation, hyperoxia induced by supplemental oxygen therapy, sepsis, various exposures and comorbidities in the NICU) insults can impact lung development. (31, 34, 36) These insults trigger the release of free radicals and inflammatory cytokines, which play an important role in the pathogenesis of BDP. (34, 37) Consistently, studies have shown that TNF-α, IL-1β, and IL-6 were significantly increased in serum and tracheal aspirate from infants who developed BPD. (38) Inflammation acts by directly inhibiting alveolar and capillary formation in the fetal lungs. The simplification of the vasculature and the concomitant alteration of alveolarization are hallmark histological features of BPD. (29, 34)

The normal alveoli and capillary network develop and mature from approximately 36 weeks of gestation until the age of 3 years in humans. (39) Indeed, the process of alveolization is a late embryologic event that continues after birth. The lungs remain in the saccular stage until 36 weeks of postmenstrual age. At this morphological stage, the conducting airway system

terminates in large saccules (undivided air spaces). At 36 weeks, the saccules subdivide, through a process of septation, into mature alveoli, the functional units of gas exchange. (29, 31, 39) Exposure to inflammation during this process can lead to disruption of alveolarization: decreased alveolar surface area, with altered septal architecture and enlarged airspaces. In other words, the lungs have fewer but larger alveoli, which is suboptimal for gas exchange and can therefore lead to respiratory failure. Inflammatory lung injury can also lead to bronchiolar and interstitial fibrosis, with compensatory emphysema of less damaged acini. (37)

Our laboratory has previously demonstrated in a mouse model that prenatal exposure to IL-1β results in lung injury with a histological phenotype similar to BPD. (25) The lungs of IL-1β-exposed pups exhibited grossly atypical lung parenchyma, characterized by disrupted alveolarization with lack of septation, resulting in a decreased number of larger alveoli. (Figure 1)

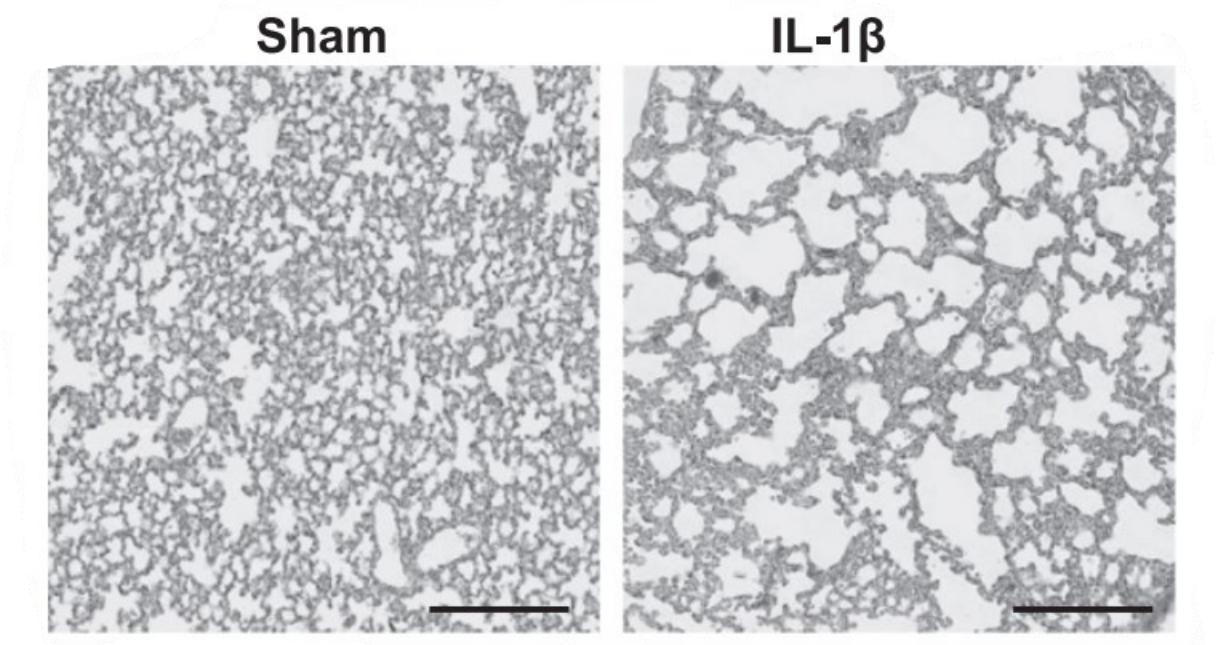

Figure 1 Lung injury in mouse offspring induced by prenatal exposure to IL-1β. Lungs were collected from adolescent mice on post-term day 15. Scale bars, 250 μm. *Reproduced with the permission of Mathieu Nadeau-Vallee. Data published in: Nadeau-Vallée M, et al. Antenatal Suppression of IL-1 Protects against Inflammation-Induced Fetal Injury and Improves Neonatal and Developmental Outcomes in Mice. The Journal of Immunology. 2017.* (25)

Patients with BPD have limited lung reserve, and often suffer from repeated infections. They may develop pulmonary hypertension which may progress to cor pulmonale. Prolonged hospitalizations, persistent respiratory disease (such as obstructive disease, bronchial hyperreactivity, asthma, and early emphysema), growth and developmental delays, and poor long-term neurodevelopmental outcomes (e.g., cerebral palsy, neurodevelopmental, cognitive, and academic impairment) are common in this population.(4, 34)

Management of BPD may include antenatal corticosteroids, postnatal surfactant, caffeine, vitamin A, avoidance of excessive fluid intake early in life, and use of the lowest possible FiO2 levels, tidal volumes and airway pressures. (40) However, unfortunately, there is still no cure for BPD. (34) Some medications, such as bronchodilators, may temporarily improve lung function, but continued administration has not been shown to have any beneficial effect. (4) Postnatal use of corticosteroids (e.g., dexamethasone or hydrocortisone) can effectively inhibit inflammatory mediators by a variety of mechanisms, but significant adverse effects (including intestinal perforation, gastrointestinal bleeding, hypertension, hypertrophic cardiomyopathy, hyperglycemia, growth retardation, and unfavorable neurodevelopmental outcomes) limit their use. (34, 41) Experts are therefore reluctant to recommend postnatal systemic corticosteroids for the treatment of BPD. Thus, the therapeutic tool for BPD remains an important unresolved issue. (34, 42)

1.4.3 Necrotizing Enterocolitis (NEC)

While techniques to aggressively manage premature infants from a pulmonary standpoint have become a priority, the necrotizing enterocolitis (NEC) incidence is rising and soon expected to supersede pulmonary insufficiency as the principal cause of death in premature infants. Indeed, NEC is a devastating inflammatory bowel disease that primarily affects infants born prematurely. NEC is also frequently reported in the literature among the early neonatal complications of intrauterine growth restricted neonates. (43, 44) Up to 11% of very LBW preterm infants are affected by NEC annually. The mortality rates for infants with the disease are reported to range from 20-30% in confirmed cases to 65% when surgery is required. (45, 46, 47) NEC currently is the most common cause of death between postnatal days 15 and 60 in preterm infants born before

28 weeks of gestation. (46) Despite the significant advances in neonatal medicine, the prevalence of NEC has not decreased and may even be increasing. (48)

NEC pathogenesis remains incompletely understood, but it is thought to be highly multifactorial. The main associated risk factors and etiologies are prematurity, IUGR, genetic predisposition, immature mucosal barrier and immune response, *in utero* bacterial infection, hypoxia-ischemia, abnormal post-natal microbial colonization, and post-natal formula feeding. There is a clear contribution of both prenatal and postnatal insults. (4, 49) The existing literature highlights an intestinal dysbiosis in NEC that results from an imbalance between proinflammatory mediators (such as TLR4, IL-1β, IL-6, TNF, and IL-18) on the one hand, and protective anti-inflammatory mediators (such as TLR9, IL-1Ra, IL-10, and TGF-β2) on the other. This imbalance leads to a vicious cycle in which excessive pro-inflammatory signaling and intestinal injury reinforce each other and perpetuate disease activity.(46, 50) Studies have shown that exaggerated signaling of TLR4 (receptor for LPS component of Gram-negative bacteria) on the intestinal epithelium plays a critical role in NEC development. TLR4 is expressed at higher levels in the intestinal epithelium of premature infants compared with that of full-term infants. and its activation by luminal bacteria in turn activates Nlrp3 inflammasome and subsequently release IL-1β. This unbridled inflammation leads to mucosal death and bacterial translocation.(51) It is worth mentioning that toll-like receptors (TLRs) are vital components of the intestinal innate immune system. TLRs function by recognizing unique structural patterns conserved in pathogens and activating downstream transcription factors to mount an effective immune response. Under normal conditions, TLR activity is balanced, so that commensal gut bacteria are tolerated, while still providing immune activity against invading pathogens. In NEC, there is exaggerated activation of TLRs (primarily TLR4) leading to pathologic inflammation, local tissue injury, and a widespread inflammatory response. Tight regulation of TLRs signaling and underlying cytokines production including IL-1β is essential to regulate intestinal inflammation and to maintain the health of the developing intestinal tract. (48)

NEC will typically strike within the first two weeks of life. (52) The clinical presentation of NEC is characterized by nonspecific signs of gastrointestinal dysfunction. These signs may include abdominal distension, feeding intolerance, vomiting and hematochezia. It may progress suddenly

to pneumoperitoneum, perforation, systemic shock, overwhelming sepsis, and rapid death in severe cases. The pathognomonic feature is pneumatosis intestinalis on abdominal radiograph, which represents air trapping within the intestinal wall du to bacterial fermentation. (4, 51, 52)

NEC involves predominantly the terminal ileum and proximal colon. It is characterized in its severest form by extensive hemorrhagic inflammatory necrosis. Histopathologic landmarks include mucosal edema, hemorrhage, coagulation necrosis, bacterial proliferation, and mucosal ulceration. This disease can have focal, segmental, or diffuse ulceration and necrosis. Inflammation can be limited to the mucosa and submucosa of the intestine, or progress to transmural involvement in the most severe cases, which can lead to perforation. In addition, histological lesions may include submucosal or subserosal collections of gas which is what is seen as pneumatosis on abdominal radiograph as discussed above. (49, 50, 52) Our laboratory has previously demonstrated in a mouse model that prenatal exposure to IL-1β results in intestinal morphological abnormalities including abnormal shortening in villi. (25) The intestines of IL-1β-exposed offspring exhibited an increased incidence of villous atrophy in the jejunum-ileum. (Figure 2) Histologic findings of non-lethal enterocolitis may include this compromised villous integrity consistent with an atrophic intestinal phenotype similar to human inflammatory bowel diseases such as celiac disease or autoimmune enteropathy that are not devoid of consequences.(53, 54)

The prognosis for NEC infants remains grim and those who survive commonly must deal with long-term complications such as poor growth, cholestasis, short bowel syndrome, and neurodevelopmental delays. Some may even require gut transplantation. (46, 55) Current management of NEC is limited to bowel rest (with cessation of enteral feeds, institution of nasogastric suction), broad spectrum parenteral antibiotics, and supportive therapy (fluid resuscitation, parenteral nutrition). Treatment can imply surgery for perforation (which occurs in ~20–50% of NEC infants) or for resection of necrotic bowel. (46, 52) Therapeutic strategies have so far mainly been targeted in the postnatal period. However, there is emerging evidence suggesting that preventive interventions in the prenatal period could be of great interest to modulate the pathogenesis of NEC and prevent its devastating consequences. (51) Indeed, the persistently high mortality of NEC reveals an urgency to approach the disease differently. (51)

Development of new therapeutic strategies for NEC remains an urgent unmet need. A window of opportunity may exist *in utero* to counteract gut dysbiosis and excessive inflammation leading to morbid intestinal injuries.

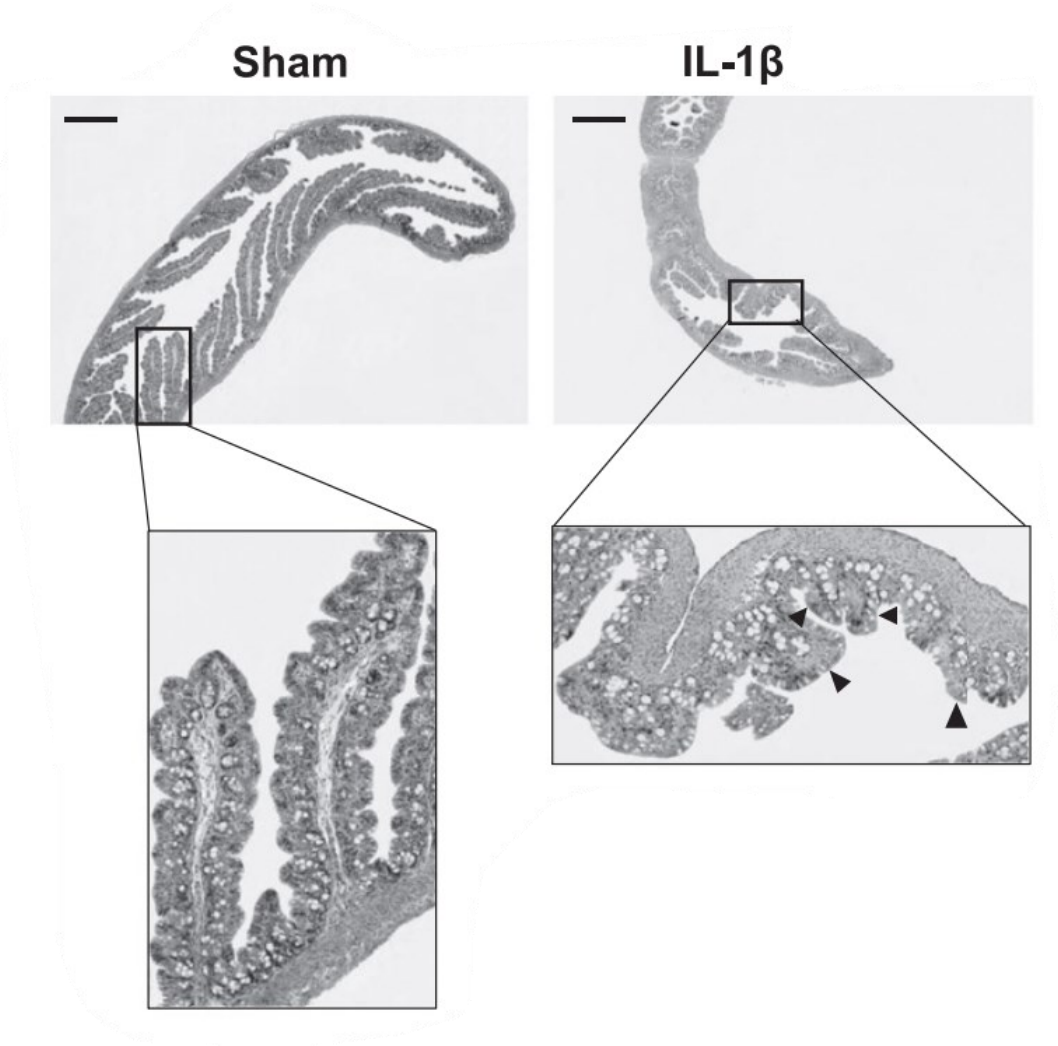

Figure 2 Intestinal injury in mouse offspring induced by prenatal exposure to IL-1β. Intestines were collected from adolescent mice on post-term day 15. Atrophied villi in IL-1β-exposed pups are indicated by black arrows. Scale bars, 1000 mm. *Reproduced with the permission of Mathieu Nadeau-Vallee. Data published in: Nadeau-Vallée M, et al. Antenatal Suppression of IL-1 Protects against Inflammation-Induced Fetal Injury and Improves Neonatal and Developmental Outcomes in Mice. The Journal of Immunology. 2017*. (25)

1.4.4 Neonatal Encephalopathy (NE): Periventricular Leukomalacia (PVL) and Intraventricular Haemorrhages (IVH)

Neonatal cerebral injury mainly affect infant born before 32 weeks of gestation and/or with a birth weight of less than 1500 g. (56) Brain injury has also been strongly associated with intrauterine growth restricted infants and is known to be a major contributing factor to perinatal morbidity and mortality worldwide. (57) IUGR accounts for ∼40% of neurologically damaged children. (58) The neuropathology of neonatal cerebral injury reports multiple lesions mainly including periventricular leukomalacia (PVL) and intraventricular haemorrhages (IVH). The complex perinatal brain injury phenomenon can be brought together under the general term: neonatal encephalopathy (NE). (56) IVH and PVL are often co-occurring characteristics of brain injury in preterm infants. (59)

PVL consist in white matter injury around the ventricles of the brain. The initial presentation of PVL is classically silent and evolves over weeks. Clinical manifestations can include spastic diplegia or tetraplegia (a type of cerebral palsy caused by damage of motor corticospinal tract axons), visual impairment (caused by damage of the optic radiations), and cognitive deficits (caused by cortical lesions). Developmental delays are usually increasingly apparent over time. Diagnosis of PVL requires neuroimaging (i.e., cranial ultrasound [US] or magnetic resonance imaging [MRI]).

IVH is also most often clinically silent. The specific pathophysiology of IVH involves the fragility of immature vessels highly susceptible to hypoxia-ischemia and inflammation at this early stage of brain development, making them vulnerable to rupture. (60) When severe, clinical signs such as apnea, bradycardia, general weakness, seizures, and bulging of the fontanelles can be observed. It is usually detected in the first 72 hours of life by screening transfontanella US performed routinely in NICUs. IVH represents an independent risk factor for neurosensory and behavioral developmental disorders. (60)

The etiology of NE is complex and multifactorial. The pathogenesis is a combination of destructive phenomenon, by inflammatory, ischemic, and oxidative damage, with impaired cellular maturation of microglia and oligodendrocyte. (56) There is a known intrinsic vulnerability of cerebral white matter du to the high metabolic demand of early differentiating oligodendroglial

cells. Compelling evidence supports the fundamental role of inflammation in the pathogenesis of NE. Accordingly, a wide range of perinatal triggers of inflammation such as hypoxia-ischemia from inadequate perfusion, mechanical stress, oxygen toxicity, infections (including chorioamnionitis) have been strongly correlated with PVL and cerebral palsy. (61) Similarly, pre-exposure of the fetus to adverse intrauterine conditions, as in IUGR, results in an increased frequency of intraventricular hemorrhage (IVH). (56) Prenatal and postnatal triggers synergistically cause inflammation in the fetus and neonate. Excessive systemic and central nervous system inflammation is strongly implicated in the disturbances to neuronal and oligodendrocyte development. This can result in an important reduction in brain growth that leads to reduced white and grey matter volumes. Among all the proinflammatory mediators involved, IL-1 stands out significantly. A systematic review including 47 studies found that 100% of infants with neurological injuries had elevated levels of IL-1β in their cord blood. (62) High levels of IL-1β have been associated with white matter inflammation and injury in post-mortem brain tissue sections from human preterm infants. (63). Moreover, increased production of the IL-1β secondary to polymorphisms in the IL1B gene was associated with an increased risk of IVH and PVL. (64) Consistently, in animal models of perinatal brain injury, elevated systemic and cerebral IL-1β was strongly associated with white matter injury. (65, 66, 67)

It is interesting to note that another important contributing mechanism of NE in PTB is premature inhibition of cerebral angiogenesis. There is significant fetal angiogenesis from 24 to 32 weeks of gestation. Accordingly, significant human brain growth occurs during the third trimester, with a doubling of whole brain volume. (68) This enhanced neurogenesis requires enhanced angiogenesis. Cerebral angiogenesis is mediated via vascular endothelial growth factor (VEGF) and angiopoietin-2 both present in high levels antenatally. This angiogenesis naturally decreases after birth due to the relative postnatal hyperoxic environment that results in down-regulation of VEGF. Thus, in the context of PTB, immature vessels, still dependent on VEGF, will be obliterated postnatally following the abrupt premature withdrawal of VEGF. The resulting ischemia leads to neuronal apoptosis, which in turn can lead to white matter injury, PVL, and decreased brain volume. (60) Correspondingly, previous data from our laboratory showed that prematurity,

induced in mice by antenatal exposure to IL-1β, resulted in a diffuse microvascular degeneration at 15 days of life. (Figure 3)

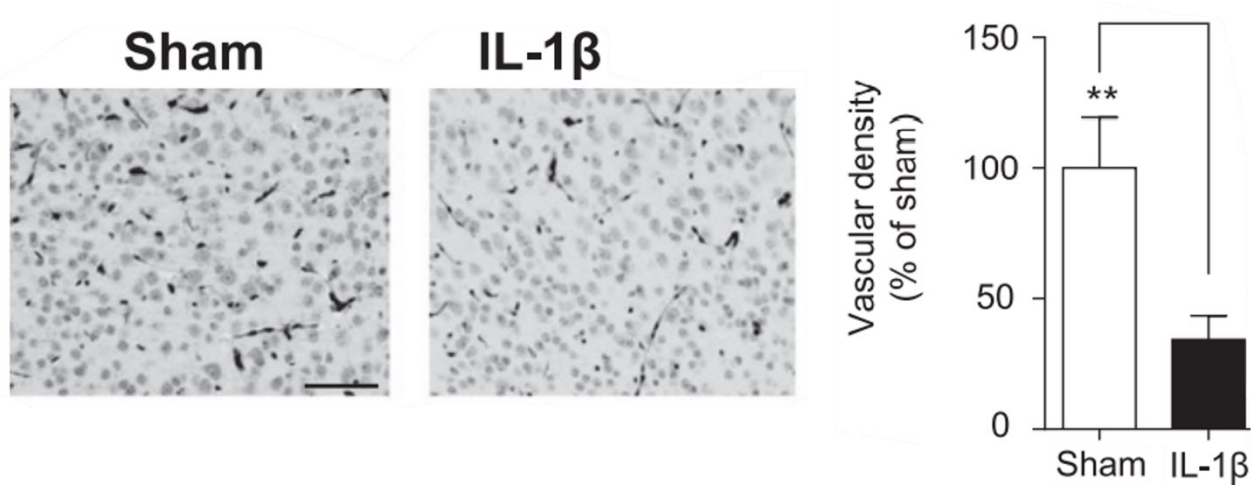

Figure 3 Brain injury in mouse offspring prenatally exposed to IL-1β. Brains were collected from adolescent mice on post-term day 15. Vasculature is stained with lectin. Vascular density is dramatically decreased in the cerebral cortex of mice offspring antenatally exposed to IL-1β. Scale bars, 100 µm. *Reproduced with the permission of Mathieu Nadeau-Vallee. Data published in: Nadeau-Vallée M, et al. Antenatal Suppression of IL-1 Protects against Inflammation-Induced Fetal Injury and Improves Neonatal and Developmental Outcomes in Mice. The Journal of Immunology. 2017 (25)*

Other histopathological features may include hypomyelination, gliosis, decreased white matter mass, dilatation of the lateral ventricles, and atrophy of the corpus callosum, varying according to the severity of PVL. (56)

NE is responsible for severe irreversible disabilities such as cognitive deficits, behavioral and attentional disorders, socialization defects, and significant motor sequalae, including cerebral palsy. (56, 69) Sensory deficits are also reported du to damage to visual, auditory, and somesthetic fibers. Hence, the cumulative economic cost of disability associated with perinatal brain injury is substantial and continues to rise. It is estimated that cerebral palsy alone costs society more than $11.5 billion per year in the United States. Prevention of NE and its associated disabilities would significantly reduce this socioeconomic burden. (65)

There are currently no validated therapeutic to treat PVL or IVH or their irreversible brain damage. (56) Initial management consists primarily of maintaining cerebral perfusion, oxygenation, and ventilation with attention to avoiding abrupt changes in systemic hemodynamics. Secondary management also includes early identification of cognitive, motor, or sensory consequences in order to initiate rehabilitative therapies (such as physical, occupational, speech, and visual therapy).

Some medical interventions have shown positive therapeutic effects in preventing adverse neurological outcomes. For example, mild therapeutic hypothermia, via whole body or head cooling, was shown to improve infants' survival without disability. However, this therapeutic strategy is far from being miraculous, as nearly half of all infants still dies or survives with disabilities. (70) Another example, is antenatal maternal administration of magnesium sulfate (MgSO4) and corticosteroids (intramuscular betamethasone). These molecules are usually administered to women in preterm labour between 24-34 weeks of gestation to respectively calm uterine contractions and stimulate fetal lung maturation. They both have shown desirable neuroprotective properties as MgSO4 reduces the risk of cerebral palsy and motor dysfunction and corticosteroid reduces the risk and the severity of IVH in neonates. (60) (65) Unfortunately, most of the recent studies that tested the potential of MgSO4 for perinatal neuroprotection were relatively underpowered, and suggested that any improvements in neurodevelopment were at best modest or absent. (70)

IVH and PVL are of great concern to neonatologists, as they are responsible for a significant proportion of neonatal deaths and permanent long-term adverse neurodevelopmental outcomes. Although much progress has been made in the management of preterm and growth-restricted neonates, there remains an urgent need to develop more effective therapies to better protect the infant brain from harmful perinatal inflammation.

The significant role of prenatal inflammation in IUGR, PTB, and neonatal morbidities will be discussed in detail in the next section.

2. Inflammation and its Role in IUGR and PTB

In order to properly address the role of inflammation in these two pathological conditions of interest, it is important to establish its physiological role in a normal pregnancy with full-term delivery.

2.1 Physiological Inflammation in Pregnancy and Uterine Transition Towards Birth

Inflammation is critical to the success of female reproduction, every step of the way from menstruation to childbirth.

In early pregnancy, inflammation is essential to embryo implantation, trophoblast invasion, decidualization and placentation. (19, 71) The important remodeling process of the endometrium and myometrium in preparation for pregnancy are made possible by the recruitment of immune cells at the site of implantation, including natural killer (NK) cells, macrophage (specially M1 phenotype), and lymphocytes (specially T-helper 1 cells). These immune cells regulate the inflammatory environment through the secretion of pro-inflammatory cytokines like TNFα, IL-6, IL-8, IL-15, GM-CSF, CXCL1, CCL4. Decidualized stromal cells also secrete several growth factors and cytokines (including IL-11, epidermal growth factor [EGF], heparin-binding EGF-like growth factor, prolactin and insulin-like growth-factor-binding protein-1). Notably, high levels of a pleiotropic cytokine from the IL-6 family, the leukemia inhibitory factor (LIF), have been reported in uterine tissues at the implantation stage. LIF plays an indispensable role in the regulation of immune response in the uterus in early pregnancy. It recruits specific leukocyte subpopulations to the site of implantation. It is involved in the regulation of multiple processes including the transformation of the uterus into a receptive state, decidualization, embryo-endometrial interaction, and trophoblast invasion. LIF expression and production is known to be induced by pro-inflammatory cytokines such as IL-6 and IL-1β. (72, 73) The creation of this early inflammatory environment is necessary for the success of the pregnancy, as absence of inflammation and appropriate remodeling process of the uterine wall and spiral arteries can actually lead to spontaneous abortion at this early stage. (74) Similarly, an excessive immunosuppression and anti-inflammatory state can lead to repeated pregnancy failures

secondary to implantation failure, for example in women receiving treatment for autoimmune diseases such as Crohn's disease or rheumatoid arthritis. However, an excessive inflammatory state can also lead to early pregnancy loss. In fact, pregnancy requires a fine balance between pro-inflammatory and anti-inflammatory influences. (75)

Gradually, between 4 to 9 weeks of gestation, signals from the embryo will start inhibiting the expression of pro-inflammatory cytokines and chemokines and will shift the phenotype of local macrophages (from M1 to M2). M2 macrophages begin secreting anti-inflammatory cytokines like IL-10. The new homeostatic anti-inflammatory microenvironment generated by the crosstalk between the fetal-placental and the maternal immune system creates an optimal setting for healthy fetal growth. (76) The anti-inflammatory state remains for the longest period of the pregnancy (lasting from week 13 to week 27).

At this stage, pregnancy really becomes a unique immunological condition. On one hand, there is the essential establishment of immune tolerance to the semi-allogenic fetus (which presents paternal antigens that could be considered as "foreign" tissue by the maternal immune system and lead to "rejection" of the fetus). On the other hand, preserving an active protective mechanism against microbial infections remains essential. Among the contributing mechanism, the shift from cellular immunity (driven by T-helper 1 [Th1] activity) to humoral immunity (driven by T-helper 2 [Th2] activity) and the expansion in regulatory T cells (Treg) population will both encourage maternal tolerance of the fetal antigen allograph, while maintaining protection against pathogens (74, 77, 78). Simply put, the immune environment and inflammatory processes are regulated by signals coming from both the mother and the fetus, and these dynamic immunological states are continuously adapting to the stages of fetal development (as schematized in Figure 4).

It is important to note that the widespread belief that pregnancy entails an immunosuppressive state is incorrect. (71, 76) The maternal immune system is not suppressed or weaker during pregnancy, it is in fact stronger. It adapts and learns to tolerate fetal antigens. The term "immunomodulation" as opposed to "immunosuppression" would thus be more appropriate (75). In addition, from an evolutionary perspective, a weakened immune system at this crucial

stage of life would seem incompatible for the survival of the species. A pregnant female needs, more than ever, her immune system to protect herself and her fetus from invading pathogens (bacteria, viruses, parasites, or fungi) and harmful substances of our environment.

In late pregnancy, the placenta's biological clock starts ticking and sends signals to the mother to prepare her gestational tissues for labour and delivery. (71) Thus, we are witnessing a second immunological shift from an anti-inflammatory to a pro-inflammatory state. The immune system plays a critical role in parturition. In fact, parturition is the culmination of a strong physiological inflammatory process orchestrate by uterine invasion by leukocytes (macrophages, neutrophils, lymphocytes [mainly T cells]). The leukocytes will produce a broad range of chemokines and pro-inflammatory cytokines (like IL-1β, IL-6, IL-8, TNF-α), as well as prostaglandins, PTGS2, and extracellular matrix remodeling enzymes (like MMP9). (79) This process will lead to uterine activation (beginning of irregular contractions) with cervical ripening (dilatation and effacement of the cervix due to changes in cervical composition) and weakening of fetal membranes. There will be a detachment of the chorioamniotic membranes from the decidua and the fragilized membrane will eventually rupture. Myometrial contractility then intensifies (shifting from irregular contractions to functional contractions) and will culminate in powerful contractions to expulse the conceptus. (19, 80)

In short, inflammation (in the right dose and timing) is essential to a successful pregnancy and birth. Pregnancy is a unique immunological condition. The cross-communication between the fetoplacental and maternal immune systems allows for a strict regulation of inflammatory processes during the different stages of a healthy pregnancy.

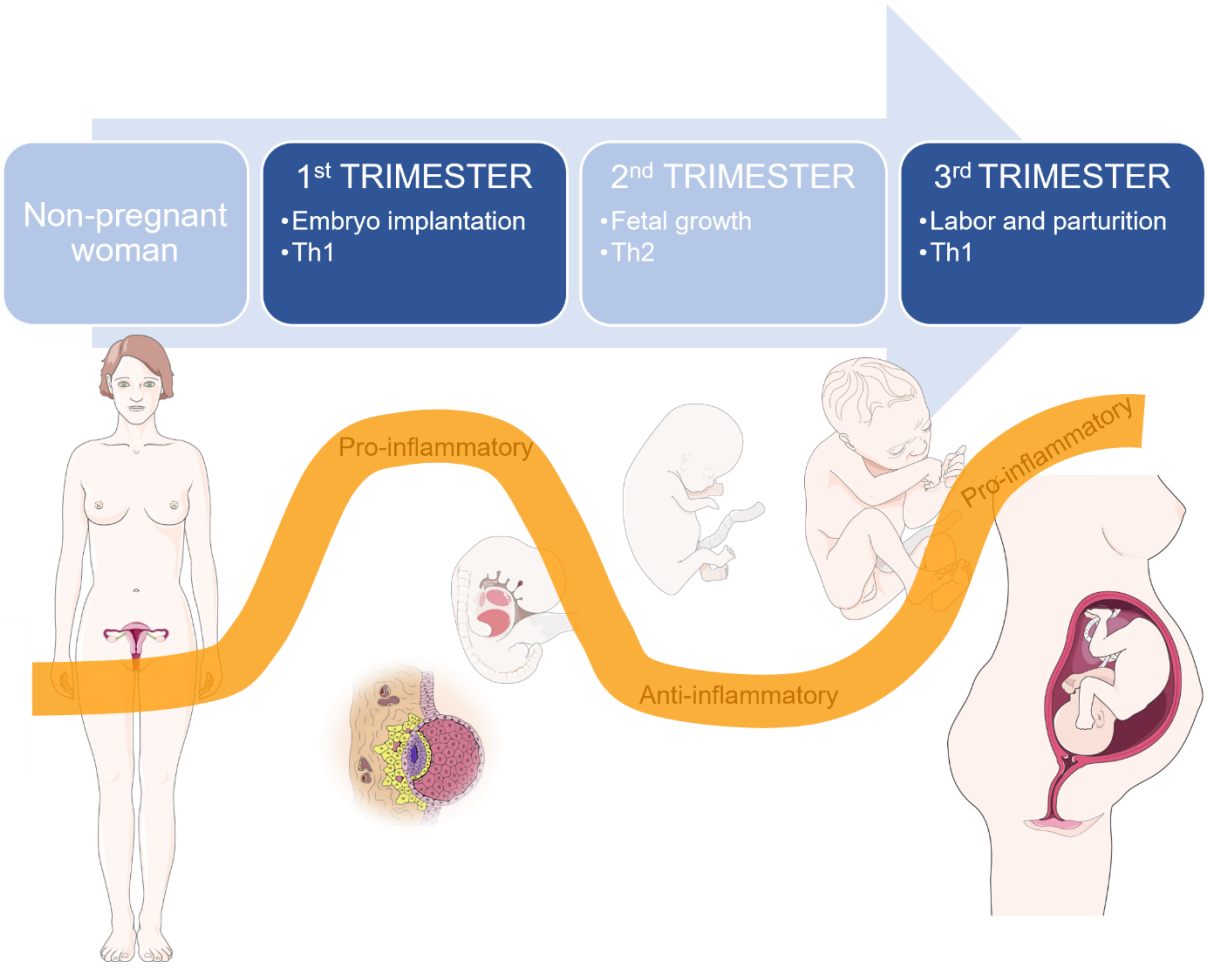

Figure 4 Differential physiological inflammatory profile according to the stages of pregnancy. The first trimester is characterized by a pro-inflammatory environment mediated by cellular immunity (Th1 type response) for successful embryo implantation. The second trimester is characterized by an anti-inflammatory environment with humoral immunity (Th2 type response) for adequate fetal tolerance, development, and growth. The third trimester is characterized by labour and parturition which are massive inflammatory processes (with Th1 type response). *The conception of this figure was inspired by Mor G, Aldo P, Alvero A. The unique immunological and microbial aspects of pregnancy. Nature reviews Immunology. 2017;17. (71) This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.*

2.2 Pathological Inflammation in IUGR and PTL

Although inflammation is necessary for successful reproduction, untimely activation of inflammatory processes can have devastating effects on pregnancy outcomes including abortion, placental dysfunction, intrauterine growth restriction (IUGR), pre-eclampsia and preterm labour. (19) As Dr Gil Mor would say: “Everything is timing in pregnancy!”. When there is excessive inflammation at the wrong time, triggered by an infectious or traumatic insult, by exposure to toxic substances (like pollution, alcohol, or tobacco) or any biological stressor, there can be a significant impact on fetal growth and eventually on premature activation of the myometrium leading to preterm labour and birth. (78)

More precisely, inflammatory stimuli consist in damage-associated molecular patterns (DAMPs) for sterile inflammation and pathogen-associated molecular patterns (PAMPs) for infectious invaders. These molecular pattern entities can activate innate immunity via pattern recognition receptors (PRRs), mostly Toll-like receptors (TLRs). TLRs 1–11 (a class of phylogenetically conserved receptors) are ubiquitously expressed by mammalian cells and will also be expressed abundantly in the decidua, placenta, and membranes throughout pregnancy. (19) Their activation leads to cytokine and chemokine production (including interleukin [IL]-1, IL-6, IL-8 and TNFα) as well as to leukocyte activation and migration to gestational tissues. As more leukocytes (macrophage, neutrophil, T cells) invade the uterus, the inflammatory response is amplified through increased secretion of pro-inflammatory mediators. (80)

Maternal inflammation can reach the fetus as pro-inflammatory cytokines are able to cross the placenta and create a toxic inflammatory environment for the developing fetus. Data supports the role of abnormal immune activation in mediating impaired fetal growth. (81) Studies have shown an increase in the maternal plasma levels of inflammatory markers (like HMGB1, circulating cell-free DNA, circulating mitochondrial DNA, uric acid), in pregnant women with IUGR. (19) In a sheep model, it was shown that sustained systemic maternal inflammation, induced by repeated intravenous exposure to lipopolysaccharide (LPS, an endotoxin produced by most Gram-negative bacteria and widely used in research for its ability to stimulate the immune system) in the early third trimester, resulted in marked intrauterine growth retardation. (82) Total circulating leukocytes and TNFα were elevated in IUGR fetuses. Other similar studies in multiple animal

models showed that IUGR fetuses exhibit increased circulating leukocyte and cytokine concentrations. (83, 84) Studies on human IUGR infants cord blood showed elevated levels of TNFα, IL-6, and IL-18. (85, 86) This pathological inflammatory state can potentially have serious consequences after birth. Indeed, fetal exposure to an intrauterine pro-inflammatory environment, while vulnerable and unprepared, can lead to serious neonatal complications such as brain damage, necrotizing enterocolitis and bronchopulmonary dysplasia (as discussed in the previous section: frequent complications of IUGR and PTB). (78)

In the context of untimely immunological activation, pro-inflammatory cytokines can directly trigger the transition from a uterine quiescent state to a subsequent unscheduled activation of the uterus (cervical ripening, weakening of fetal membranes and myometrial contractility intensification) resulting in preterm labour and prematurity. (87) The same physiological inflammatory pathways of labour (discussed in the section above: inflammation in physiological pregnancy) are triggered, but at the wrong time, i.e. too early. This results in the production of a broad range of cytokines (IL-1β, IL-6, TNF-alpha), chemokines (IL-8) and uterine activation proteins (like OXTR, COX-2, CX-43, MMPs, ET-1, iNOS, CCL2), sometimes referred to as uterotrophins. (80) Increased maternal levels of IL-1β and IL-6 and low levels of IL-10 have been reported to be associated with PTB. (88) In a study conducted by Dr Roberto Romero, of Wayne State University, USA, on a prospective cohort of women who presented with spontaneous preterm labour and whose amniotic fluid was analyzed, the frequency of intra-amniotic inflammation (diagnosed according to high amniotic fluid IL-6 concentration) was up to 70% in women who delivered before 34 weeks of gestation (moderate to late PTB) and up to 85% in women who delivered before 30 weeks of gestation (very PTB). (89) This study suggests that, despite the broad etiology of prematurity, inflammation plays an important role in the pathophysiology of a large proportion of PTBs, and it is even thought to be responsible for the majority of early preterm births. (79)

Therefore, inhibiting the inflammatory cascade appears as a promising therapeutic strategy for the prevention of intrauterine growth restriction, preterm delivery and neonatal complications. However, as described above, a very wide range of pro-inflammatory and pro-labour mediators

are involved in the cascade resulting in obstetrical and perinatal complications. The next section highlights the interest in IL-1β on which this study was centered.

2.3 Significant Role of Interleukine-1β in Adverse Obstetrical and Perinatal Outcomes

As discussed above, IUGR and PTB have been firmly linked to inflammatory processes. Of all cytokines implicated in gestational inflammation and the onset of neonatal morbidities, interleukin (IL)-1 has been most extensively studied, revealing its central role.

The IL-1 family includes 11 cytokines that regulate the inflammatory response to injuries and stressors. Two major members of the family are IL-1α and IL-1β, which bind to ubiquitously expressed IL-1R1. (19) IL-1β is a potent pleiotropic cytokine and is a key regulator of the inflammatory network. It is considered to be a master cytokine in the pathogenesis of several diseases (such as arthritis, gout, type 2 diabetes, dry eye syndrome and heart failure), inducing multiple pathways of inflammation. (90) Correspondingly, these common inflammatory diseases are known to respond well to IL-1 antagonization.

A broad body of evidence suggests that IL-1 has a critical role in pregnancy pathologies. Gene expression analysis identified a nearly 54-fold increased expression of IL-1β in the placental tissue of women with recurrent abortions. (91) Elevated levels of IL-1β in placental tissue have also been measured in high-risk pregnancies known to have placental dysfunction with reduced fetal movements (the latter being clinically associated with IUGR, oligohydramnios, preterm labour, and other adverse obstetrical outcomes). (92, 93) A growing body of evidence highlights the role of IL-1-driven inflammation in IUGR. IL-1β is elevated in the serum of pregnant women with IUGR during the third trimester of pregnancy. (94, 95) Previous data have shown that in PTB and IUGR resulting from placental malaria infection, severe inflammation was reversed by Anakinra (a pharmacological IL-1R antagonist), which significantly improved pregnancy outcomes, suggesting that IL-1β-mediated signaling is critical in the pathogenesis of PTB and IUGR. (96) IL-1 is in fact the first cytokine known to be implicated in the mechanism of PTB as well as in spontaneous full-term delivery in humans. Dr. Roberto Romero showed in 1991 that systemic administration of IL-1 induces preterm parturition in mice. (97) Premature delivery occurred systematically in all IL-1-

injected mice. (97) Similar results were obtained in non-human primates models: IL-1β intra-amniotic infusion induced preterm contractions in pregnant rhesus monkeys. (98) Preterm labour has been associated with increased levels of IL-1β in the human cervix, myometrium, fetal membranes, and amniotic fluid. (80, 99, 100)

Moreover, an elevated IL-1β blood concentration has been reported in human pre-term neonates. (101) IL-1 may strongly contribute to neonatal pathologies associated with PTB and IUGR. Studies have shown that IL-1 plays an important role in the pathophysiology of bronchopulmonary dysplasia (BPD). Tracheal aspirate samples from premature infants with respiratory distress who developed BDP had elevated levels of IL-1β. (38) Preclinical studies have shown that inhibition of IL-1 by Anakinra holds great promise for preventing BPD in growth restricted newborns. (95) Increased systemic and tissue levels of IL-1β are associated with greater risk of postnatal lifelong neurodevelopmental disorders such as cerebral palsy. (65, 67) IL-1β is considered as the main isoform implicated in neural injury. Elevated systemic and cerebrospinal IL-1β on the first days of life were associated with impaired cerebral metabolism and developmental delay at 2 years of age. Increased level of IL-1β is associated with higher risks of intraventricular hemorrhage (IVH) and periventricular leukomalacia (PVL). (65) A study of intestinal tissue from premature infants (from surgical pathology archives) revealed increased IL-1β mRNA levels in the mucosa of specimens with acute necrotizing enterocolitis (NEC). (102) BPD, IVH, PVL, and NEC, which are devastating complications of IUGR and PTB, were discussed in a more thorough manner in the previous section (Frequent Complications of IUGR and PTB). This paragraph highlights the recurrent role of IL-1 in these neonatal pathologies.

Overall, IL-1 is a potent cytokine, central to many inflammatory processes. It is considered to be one of the key mediators of preterm labour and can have major detrimental effects on the fetus. Our laboratory focusses on IL-1 as the primary target in its efforts to prevent IUGR, PTB and adverse neonatal outcomes.

To summarize, regardless of the cause of IUGR and PTB, they all share many common pathophysiological pathways in the activation of common downstream cellular and molecular inflammatory effectors including IL-1. Thus, targeting the molecular mechanisms that trigger the

onset and process of fetal tissues inflammation, growth restriction, rupture of membranes and uterine contractions may lead to therapeutic treatment and interventions that are vital to the health of future infants.

3. Existing Therapeutic Strategies

As discussed in the section above, there is unequivocal need to tackle inflammation to treat IUGR, PTB and neonatal injuries. Unfortunately, the available therapies are currently insufficient and do little to control the deleterious inflammatory cascade.

3.1 Aspirin and Management of IUGR

No pharmacological intervention has been proven to be beneficial to treat IUGR.

However, cumulative data from several trials and meta-analyses on low-dose Aspirin (acetylsalicylic acid, ASA) suggest some preventive benefit in patient at risk of IUGR. (103, 104, 105, 106, 107) The precise mechanism by which low-dose aspirin exerts preventive benefits in patient at risk of IUGR is uncertain. Aspirin is a nonsteroidal anti-inflammatory drug (NSAID). At low dosage (like the 81 mg/day used clinically in pregnant women), it inhibits preferentially the cyclooxygenase enzymes COX-1 over COX-2, which results in decreased synthesis of thromboxane A_2 (TXA2) a potent vasoconstrictor and pro-coagulant agent. (108) The hypothesis is that Aspirin improves placental perfusion, preventing preeclampsia and promoting normal fetal growth. (109) According to the 2013 Canadian clinical practice guideline approved by the Executive and Council of the Society of Obstetricians and Gynaecologists of Canada (SOGC), low-dose Aspirin should be recommended to women at risk of IUGR (e.i. with a previous history of placental insufficiency syndromes including IUGR and preeclampsia, or with a combination of other moderate risk factors like pregestational hypertension, pregestational diabetes mellitus [type I or II], obesity, advanced maternal age [AMA, > 40 years], use of artificial reproductive technology, multiple gestation, history of placental abruption or infarction). It should be initiated between 12 and 16 weeks of gestation and continued until 36 weeks of gestation. (110) Nevertheless, Aspirin is far from being a miracle cure. In a recent study on pregnant women at risk (with previous history of pre-eclampsia), there was no significant difference in the rate of IUGR between the Aspirin group and placebo group (with 27.9% and 25.6% rate of IUGR respectively). (111) In any case, there is no evidence to support the use of antithrombotic agent heparin for the prevention or treatment of IUGR. (110) Also, low-dose Aspirin prophylaxis is not recommended for prevention of fetal growth restriction, in the absence of risk factors for preeclampsia, according to The American

College of Obstetricians and Gynecologists (ACOG) recommendations. (109) There is currently insufficient evidence of the benefits of low-dose Aspirin for prevention of recurrent fetal growth restriction.

In Canadian clinical practice guideline (110), IUGR management mainly implies:

1. Investigations: clinicians should consider amniocentesis and a TORCH screen to eliminate genetical or infectious etiologies.

2. Maternal management: clinicians should monitor for preeclampsia, consider low-dose aspirin if the patient is at risk for preeclampsia, and advise smoking cessation if applicable.

3. Fetal management: serial fetal monitoring with ultrasound evaluations (usually every 2 weeks). If growth plateaus and signs of fetal distress appear, clinicians should consider inducing delivery (sometimes by cesarian section, in cases of abnormal fetal heart rate or malpresentation).

IUGR remains a problem associated with significant perinatal morbidity and mortality. Further research is needed to better define optimum management of IUGR and to improve the therapeutic arsenal.

3.2 Tocolytics and Management of PTB

When it comes to PTB, there is great concern over the lack of effective and safe treatment options for the management of preterm labour. (112) At present, treatments employed in the management of PTB are called tocolytics. Most tocolytic agents act by directly suppressing uterine contractility. They can extend gestation by at best approximately 1 week. They provide a short window of opportunity for the care providers to prepare for the birth of the preterm infant (e.g., by administering betamethasone for fetal lung maturation or by transferring the patient to high-level care facilities), but they rarely effectively reduce neonatal morbidities in clinical practice. In fact, studies have shown that tocolytic drugs are not associated with significant improvement in perinatal or neonatal outcomes and have adverse effects on women in preterm labour. (113, 114) Current practice in Canada includes the "off-label" use of magnesium sulfate, indomethacin, nitroglycerin, and nifedipine in attempt to treat preterm labour.(112)

3.2.1 Betamimetics

Betamimetics (Ritodrine, Terbutaline, Salbutamol) are the first tocolytics studied. (115) They function as beta-2 adrenergic receptor agonists. They act on smooth muscle tissues and inhibit myometrial contractions. They provide at best a 48-hour delay of delivery. In one of the largest randomized controlled trials of tocolytics, the Canadian Preterm Labour Trial compared ritodrine with placebo and found no significant beneficial effect on perinatal mortality or pregnancy prolongation. (114) As beta-adrenergic activity is responsible for a wide range of homeostatic functions, Tocolytics present extensive side effects including tachycardia, hypotension, arrhythmias, dyspnea, bronchodilation, pulmonary edema, tremors, anxiety, headaches, nausea, hyperglycemia, hypokalemia. Their side effect profile is sufficiently significant to warrant use of other therapeutics when available. (116) Therefore, the use of betamimetics is declining significantly. They are still used in USA and Asia, but Betamimetics are not used for the treatment of PTB in Canada.

3.2.2 Magnesium Sulfate

The off-labeled use of magnesium sulfate ($MgSO_4$) is widespread in North America. Its mechanism of action is not fully unraveled; magnesium regulates calcium absorption, which has an impact on

muscle contraction and neuronal activity promoting uterine quiescence. (115) There is also a known benefit to administering magnesium sulfate for fetal neurological protection. However, there are little evidence for its efficacy as a tocolytic with conflicting and generally low-power studies. Plus, there are growing concerns regarding its safety. In a study published in the American Journal of Obstetrics and Gynecology, it was reported that tocolysis with magnesium sulfate required discontinuation of treatment because of serious maternal side effects (including lethargy, respiratory distress, nausea, flushing, blurred vision) in 15% of women. (117) Magnesium sulfate is not recommended for the treatment of PTB in Canada.

3.2.3 Indomethacin

Indomethacin is part of the non-steroidal anti-inflammatory drugs (NSAIDs) family. NSAIDs have a long history of use in the prevention of PTB. The main mechanism of action of Indomethacin, like other NSAIDs, is inhibition of the cyclooxygenase (COX) enzymes. Inhibition of COX enzyme prevents the formation of prostaglandins (which are involved in facilitating uterine contractions and cervical ripening). There is little evidence to suggest that Indomethacin and other COX inhibitors should be prioritized over other available tocolytics. (115) Indomethacin did not delay deliveries for more than 48 hours, similarly to betamimetics and magnesium sulfate. In addition, the use of indomethacin in the third trimester have been associated with serious adverse effects in the fetus including oligohydramnios, and constriction of the ductus arteriosus. (118, 119, 120) Newborns exposed to indomethacin *in utero* had higher incidences of necrotizing enterocolitis (NEC), intraventricular hemorrhage (IVH), and patent ductus arteriosus (PDA). (121) Despite these life-threatening fetal complications, the off-label use of indomethacin, especially as a second line tocolytic, is widespread. Indomethacin is not recommended for the treatment of PTB in Canada.

3.2.4 Nitric Oxide Donors

Nitric oxide donors (Nitroglycerin, Glyceryl trinitrate) facilitate smooth muscle relaxation via the potent endogenous hormone nitric oxide (NO). Seems promising for the maintenance of uterine quiescence. Disappointingly, with Nitroglycerin, tocolytic failures were more common than with other tocolytics. (122) Moreover, it was reported that 25% of women required discontinuation of nitroglycerin therapy du to persistent hypotension. (122) Nitric oxide donors can have a profound

hemodynamic impact because of their potent vasodilatation effects. Nitric oxide donors are not recommended for the treatment of PTB in Canada.

3.2.5 Calcium Channel Blocker (CCB)

CCBs (Nicardipine, Nifedipine, the latter being the most extensively studied in PTL) are the most effective tocolytics available with up to 7-day delay in labour according to numerous meta-analyses. The mechanism of action of CCBs is based on its ability to block the intracellular calcium influx which inhibits myosin-actin crossbridge formation and reduces muscle contraction. (115) Side effects, such as headache and flushing, are infrequent. Data showed that less women had to discontinue treatment due to adverse effects compared to other tocolytics. (123, 124) Moreover, fewer neonatal adverse outcomes (such as neonatal respiratory distress syndrome, necrotizing enterocolitis, intracranial hemorrhage, and neonatal jaundice) have been reported with the use of Nifedipine, with associated reduction in the duration of stay in the NICU. (125) Although, a 7-day delay represents a significant increase over the 24- or 48-hour delay conferred by most other tocolytics, it would definitely be desirable in clinical practice to be able to postpone labour further. In Canada, Nifedipine is the first-line candidate for tocolysis. Even though maintenance tocolysis with CCBs is ineffective in delaying labour beyond 7 days, it remains the best treatment available to date. Further studies on the long-term use of CCBs combined with other tocolytic agents might be needed to enhance its efficacy. (125, 126)

3.2.6 Oxytocin Antagonists

Oxytocin antagonists (Atosiban) act on the myometrial cells by inhibiting the signaling cascade of oxytocin receptors (which are G-protein-coupled receptors). Atosiban thereby inhibits IP3 and diacyl glycerol (DAG) upregulation, which ultimately leads to inhibition of calcium release into the cytosol and prevention of myometrium contraction. (115) Atosiban has been proven to significantly decrease uterine contractions during PTL and prolong uterine quiescence for up to 48 hours, similar to other tocolytics. It is currently not available for use in North America as the possible association between Atosiban and death in premature infants has kept the FDA from approving the drug. (127) Studies failed to demonstrate improved neonatal outcomes compared to a placebo or other tocolytics.

3.2.7 Progesterone Analogs

Hydroxyprogesterone caproate (17α-OH-progesterone, Makena) is an analog to pro-gestational hormone P4. This hormone promotes uterine quiescence for most of pregnancy by blocking the transcription of pro-labour genes in the myometrium. There is insufficient evidence of any clinical benefit of Makena. Research on its use as a prophylactic therapeutic to prevent PTB has produced contradictory data. (128) Only one study conducted in women with singleton pregnancies and a mid-gestation cervical length greater than 25 mm resulted in a positive outcome for prophylactic therapy against PTL using a vaginal suppository. (115). In short, progesterone analogs may be effective in certain specific cases, but there is a lack of sufficient evidence.

Overall, questions on efficacy and safety of the tocolytics remain unanswered. Obstetricians and neonatologists continue to struggle with a deficient armamentarium to battle the most important cause of neonatal mortality and morbidity of this century, preterm birth. Currently tocolytics focus on myometrial contractions and have minimal impact on the inflammatory mediators involved in fetal injury. They slow down labour (a little bit), but they do not appropriately address the inflammatory cascade keeping the fetus in a hostile inflammatory environment. There is an urgent need to develop agents that can provide not only a longer-lasting delay of PTB, but also a potent anti-inflammatory effect that can clearly impact neonatal and child health.

3.3 Interleukin-1β Antagonists

Initial inflammatory events have become a novel attractive target for protecting the fetus from harmful inflammation and delaying preterm labour. An influential upstream target is IL-1β, as it is thought to be the ultimate initiator of many inflammatory processes leading to adverse obstetrical and neonatal outcomes including IUGR, PTB and neonatal morbidities such as BPD, NEC, IVH and PVL (as discussed in the section on the "Significant Role of Interleukine-1β in Adverse Obstetrical and Perinatal Outcomes").

Currently, three anti-IL-1 molecules are approved for clinical treatment of inflammatory disorders: Kineret (IL-1 receptor antagonist), Rilonacept (soluble decoy receptor), Canakinumab (neutralizing monoclonal anti-IL-1β antibody) . (90)

Kineret (Anakinra), used primarily in the treatment of rheumatoid disorders, is the most studied in PTB. It is a recombinant version of the endogenous interleukin-1 receptor antagonist (IL1-Ra). It was able to show some desirable effects mainly on fetal inflammation and injury in rodent, sheep, and nonhuman primate models, but unfortunately, it has shown very limited efficacy for preventing PTB. (129, 130, 131, 132)

The first problem with these molecules is their large molecular size. Kineret molecular size is ~17 kDa, Canakinumab is ∼150 kDa, and Rilonacept is ~251 kDa. This limits optimal bioavailability to the uteroplacental compartment. Although these biologics can be transported through the placenta by an active process involving the neonatal Fc receptor in the syncytiotrophoblast, they are not readily transported across the placenta before the organogenesis stage (before 14 weeks of gestation). (133) In addition, even after the organogenesis stage, much higher doses of Kineret were required to inhibit inflammation in the uteroplacental unit when administered systemically, whereas uteroplacental inflammatory mediators were effectively reduced when Kineret was administered at standard doses locally into the amniotic fluid of non-human primates (which would be a non-negligible medical procedure in a clinical setting). (25, 134) Similarly, a study on pregnant marmoset monkeys detected the presence of Canakinumab in the fetus when using doses 23 to 230-fold the maximum recommended human dose (MRHD). (135, 136) As such, the large molecular size of these drugs appears as a hinderance to their effective use as systemically

administered uteroplacental inflammation inhibitors because of their suboptimal access and distribution to gestational tissues.

The second main drawback of these molecules is their property of orthosteric antagonism. They competitively bind to the natural ligand's receptor site, the orthosteric binding site. As competitive antagonists, they exert non-specific inhibition of all IL-1β signaling pathways which potentially induces undesirable side effects. They inhibit among others the NF-κB pathway, which is paramount for appropriate immune vigilance. Indeed, adverse events reported with these drugs include immune suppression, infections (such as upper respiratory tract infections), gastrointestinal disorders, and vertigo. (137) Adding to this, these molecules tend to trigger undesirably frequent injection site reactions and are very costly. (138)

Given these shortfalls, another approach would be to identify new ligands that would bind to remote allosteric sites of the receptor to exert more selective inhibition of IL-1β signaling pathways. This strategy will lead to the design of a small peptide antagonist of the interleukin-1 receptor, Rytvela, created by a team of chemists from our laboratory.

4. Rytvela, a Novel IL-1β Antagonist

As discussed above, currently approved IL-1-targeting therapies have failed to show expected efficacy in pre-clinical studies of preterm labour. Confident that interleukin-1 antagonism remains a promising solution, the host lab has designed a new potent IL-1 antagonist with unique modulatory pharmacologic properties: Rytvela.

4.1 Molecular Structure

Rytvela is a small heptapeptide with a molecular size of ~0.8 kDa. It is composed of 7 amino acids: arginine €, tyrosine (Y), threonine (T), valine (V), glutamate €, leucine (L), alanine (A). Each letter of Rytvela refers to the amino acid nomenclature. It was made resistant to hydrolysis by using all d-amino acids.

Rytvela was derived from a region of the interleukine-1 receptor accessory protein (IL-1RacP). This was based on evidence that the IL-1RacP interacts with the IL-1R subunit receptor complex IL-1RI through specific regions. These regions were identified using crystallography of IL-1RI and IL-1RacP and modeling data using computational analysis (as illustrated in Figure 5) . (139) Initially, fifteen peptides were created from primary sequences of extracellular regions of the IL-1RacP. Rytvela, from the juxta-membranous region, was kept as the most potent according to our *in vitro* studies. This region of interest is remote from endogenous IL-1β-binding site (the orthosteric site), so Rytvela was confirmed to act as a non-competitive allosteric antagonist that can modulate IL-1β-binding affinity and selectively regulate downstream signaling of inflammatory pathways. (140) Details on the preserved and inhibited signaling pathways are described in the next section “Pharmacodynamic and Functional Selectivity”.

These characteristics distinguish Rytvela from the large competitive inhibitors Kineret, Canakinumab, and Rilonacept.

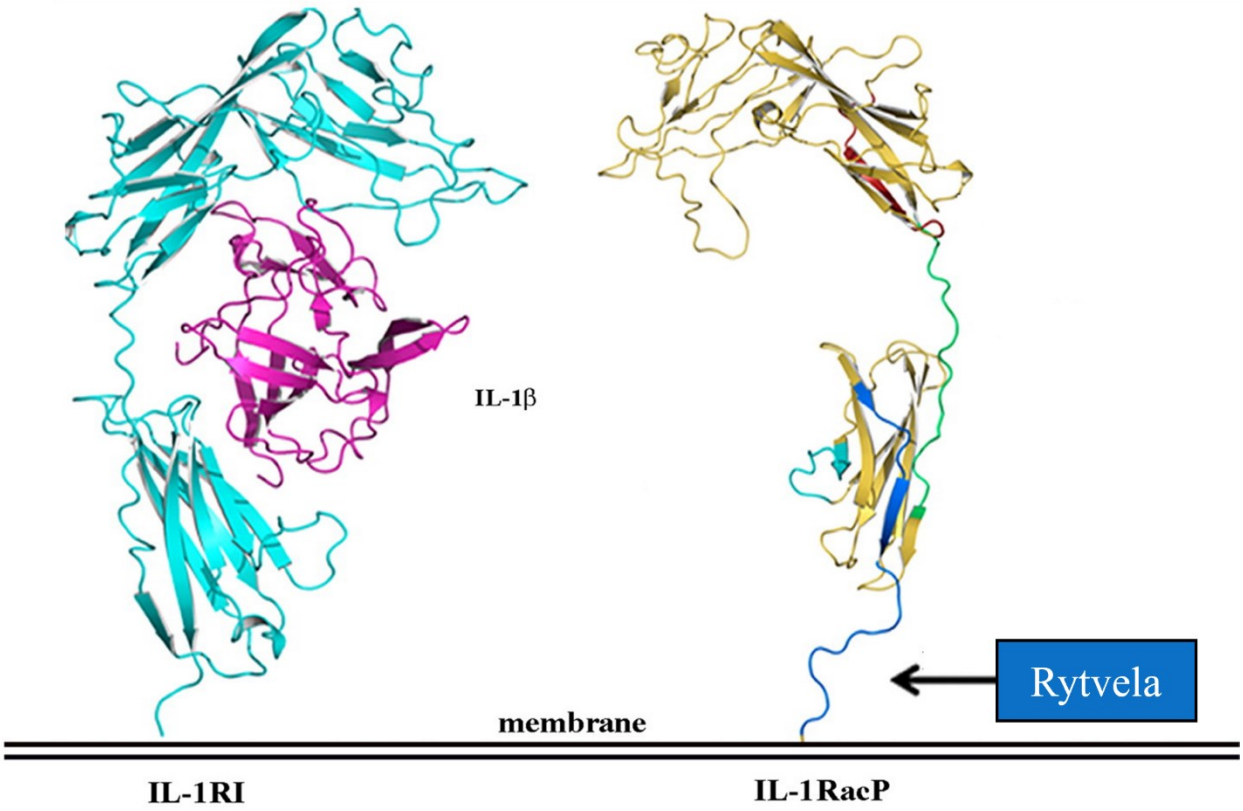

Figure 5 Ribbon-like reconstruction model of IL-1RI, IL-1, and IL-1RacP. IL-1β interacts with IL-1RI subunit. The juxta-membranous region of the IL-1RacP from which Rytvela is derived is identified in blue. *Reproduced with the permission of Christiane Quiniou. Data published in: Quiniou C, et al. Development of a novel noncompetitive antagonist of IL-1 receptor. Journal of immunology. 2008*. (139)

4.2 Pharmacodynamic and Functional Selectivity

Characterization of Rytvela revealed non-competitive antagonistic actions and functional selectivity by blocking certain IL-1R pathways without affecting others. This functional selectivity is enabled by ligands that bind in a manner that affects the dynamic conformation of the receptor to interact with its natural ligand and associated proteins required for activation of normal signaling pathways; thus, such ligands can alter signaling modalities, which may confer greater selectivity and reduce side effects, compared to orthosteric antagonists that disable all receptor-triggered functions. This desirable pharmacological characteristic can also be referred to as pharmacological permissivity (i.e., when not all functions evoked by a receptor are affected).

Rytvela moderately decreases the binding affinity of IL-1 to IL-1R but markedly depresses some of its function while preserving others; these observations contrast with those of orthosteric inhibitors such as Kineret that compete with IL-1 at the same binding site and completely block IL-1R functions.

Thus, Rytvela was shown to negatively modulate IL-1R-associated JNK, p38 MAPK, c-jun and Rho/ROCK activity, which enables it to interfere with proinflammatory gene expression and activation of both the myometrium and the macrophages. (140) However, unlike Kineret, Rytvela desirably does not inhibit IL-1β-induced NF-κB activation and monocyte-dependent phagocytosis. (Figure 6) Indeed, NF-κB is a major transcription factor that plays important physiological roles such as cytoprotection and immune surveillance, and it is thought to be particularly important in the vulnerable fetus that rely heavily on innate immunity. It has been suggested that complete blockade of NF-κB action would be undesirable during pregnancy.. (141) Correspondingly, Kineret, by interfering with all actions of IL-1R including NF-κB, increases the risk of cancer and seemingly of infections. (139) Hence, with his innovative mechanism of action, Rytvela may help decrease adverse effects such as immunosuppression which would be particularly undesirable in the context of pregnancy.

Overall, Rytvela exhibits valuable modulatory pharmacologic properties and behaves as a potent, stable, selective, and reversible non-competitive inhibitor of IL-1R. Its efficacy has been demonstrated *in vivo* in multiple models of inflammation, as described in the following sections.

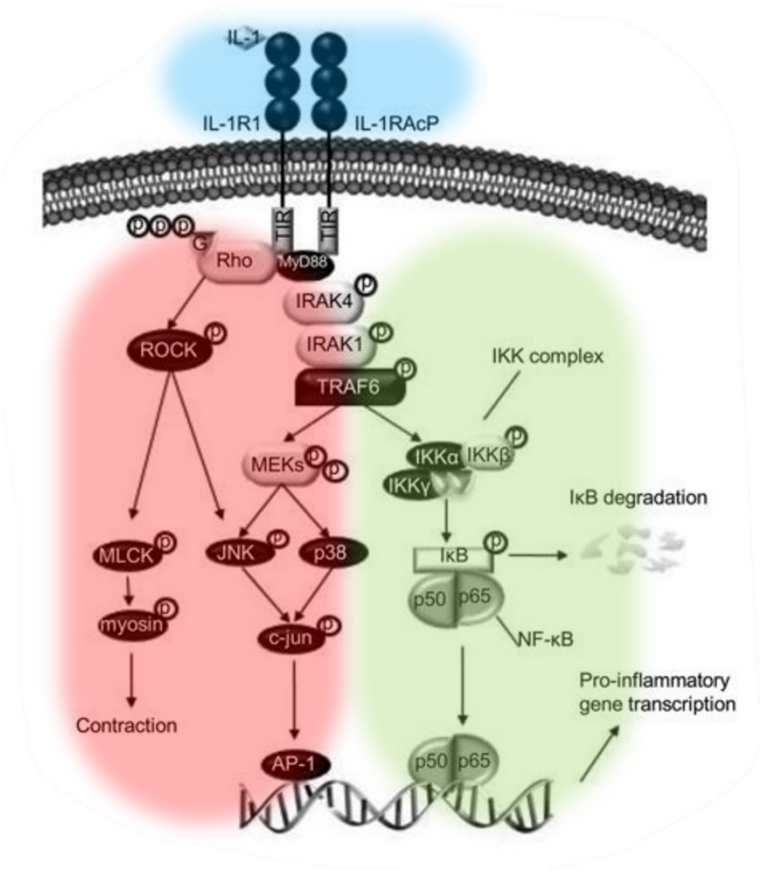

Figure 6 Simplified IL-1R intracellular signaling pathways. Rytvela binds to IL-1RI (in blue) allosterically and biased the downstream signaling pathways by selectively inhibiting SAPK/c-jun and Rho/Rho GTPase/ ROCK signaling pathways (in red), and while preserving the activity of transcription factor NF-κB (in green). *Reproduced with the permission of Mathieu Nadeau-Vallée. Data published in: Nadeau-Vallée M, et al. Novel noncompetitive IL-1 receptor–biased ligand prevents infection-and inflammation-induced preterm birth. The Journal of Immunology. 2015.* (141)

4.3 Pharmacokinetic

The full pharmacokinetics of Rytvela are still under investigation.

At this time, we have tested systemic (intravenous and subcutaneous), oral and topical administration of Rytvela, all of which have proven to be safe and effective in mouse models.

We performed a distribution study in pregnant CD-1 mice with Rytvela coupled to FITC. The results showed that Rytvela remained on the maternal side of the placenta and did not reach the fetus. We hypothesized that Rytvela reduced inflammation on the maternal side, thereby decreasing the influx of pro-inflammatory cytokines from the mother to the fetus (it is well known that pro-inflammatory cytokines can cross the placenta and induce an inflammatory response in the fetus). (25)

The elimination process of Rytvela would likely involve renal elimination or proteolysis.

4.4 Efficacy *in vivo* in models of inflammatory diseases

The efficacy of Rytvela has been demonstrated in various in vivo models of inflammatory conditions such as:

- Inflammatory bowel disease | rat model with intrarectal exposition to trinitrobenzene sulfonic acid (139)
- Contact dermatitis | CD-1 mice model with ear exposition to phorbol myristate acetate (139)
- Osteoarthritis | surgical rodent model with ligament transections (142)
- Hypoxemic-ischemic encephalopathies | Rice-Vannucci rat pup model (138)
- Ischemic retinopathy | Sprague Dawley rat pup model exposed to hyperoxia (80% O_2) (143)
- Retinopathy of prematurity | CD-1 mice pup with *in utero* exposition to IL-1β (144)
- Preterm labour | CD-1 mice pup with *in utero* exposition to IL-1β or intra peritoneal exposition to LPS or LTA (25, 145)

The last models being of more interest for this thesis, they will be further discussed in the next section.

Interestingly, in these animal models of inflammatory conditions, Rytvela was repeatedly shown to be more effective than the competitive IL-1R antagonist Kineret. (25, 139)

4.5 Efficacy *in vivo* in PTB <u>prevention</u>

In a mouse model of sterile inflammation-induced PTB (with intrauterine injection of IL-1β), prophylaxis with Rytvela has been shown to effectively prevent uteroplacental inflammatory cytokine surge. Biochemical analysis, by quantitative PCR on gestational tissues, revealed that pregnant mice exposed to IL-1 β showed a significant increase in the expression of genes encoding key proinflammatory factors (including *Tnfa, Il6, Ccl2, Ccl3, Pghs2*) in their placentas, whereas with Rytvela prophylaxis, this increase was blocked; gene expression of all cytokines remained at baseline. (Figure 7A) Prophylaxis with Rytvela prevented IL-1β-mediated induction of proinflammatory cytokines not only in the placenta but also in the uterus, amniotic fluid, and blood of the newborn. Rytvela, which can easily access the placenta, has repeatedly been shown to be more effective than Kineret in preventing upregulation of cytokine gene expression. Therefore, prophylaxis with Rytvela prevented myometrial activation and thus significantly prolonged gestation and prevented PTB whereas Kineret had no beneficial effect on gestation length. Indeed, normal gestation length in our CD-1 mouse model is 19-19.5 days. Pregnant mice exposed to IL-1β had consistent gestation lengths of under 18.5 days (considered preterm), whereas prophylaxis with Rytvela consistently prolonged gestation to over 19 days. The gestation lengths of control and treated groups were significantly longer than the inflammation-exposed group (***$p < 0.001$), whereas the group treated with Kineret exhibited no statistical difference on gestation length. (25) It is interesting to note that prolonging a mouse's gestational term by 0.5 day corresponds to an increase of approximately 1 week in humans. (146)

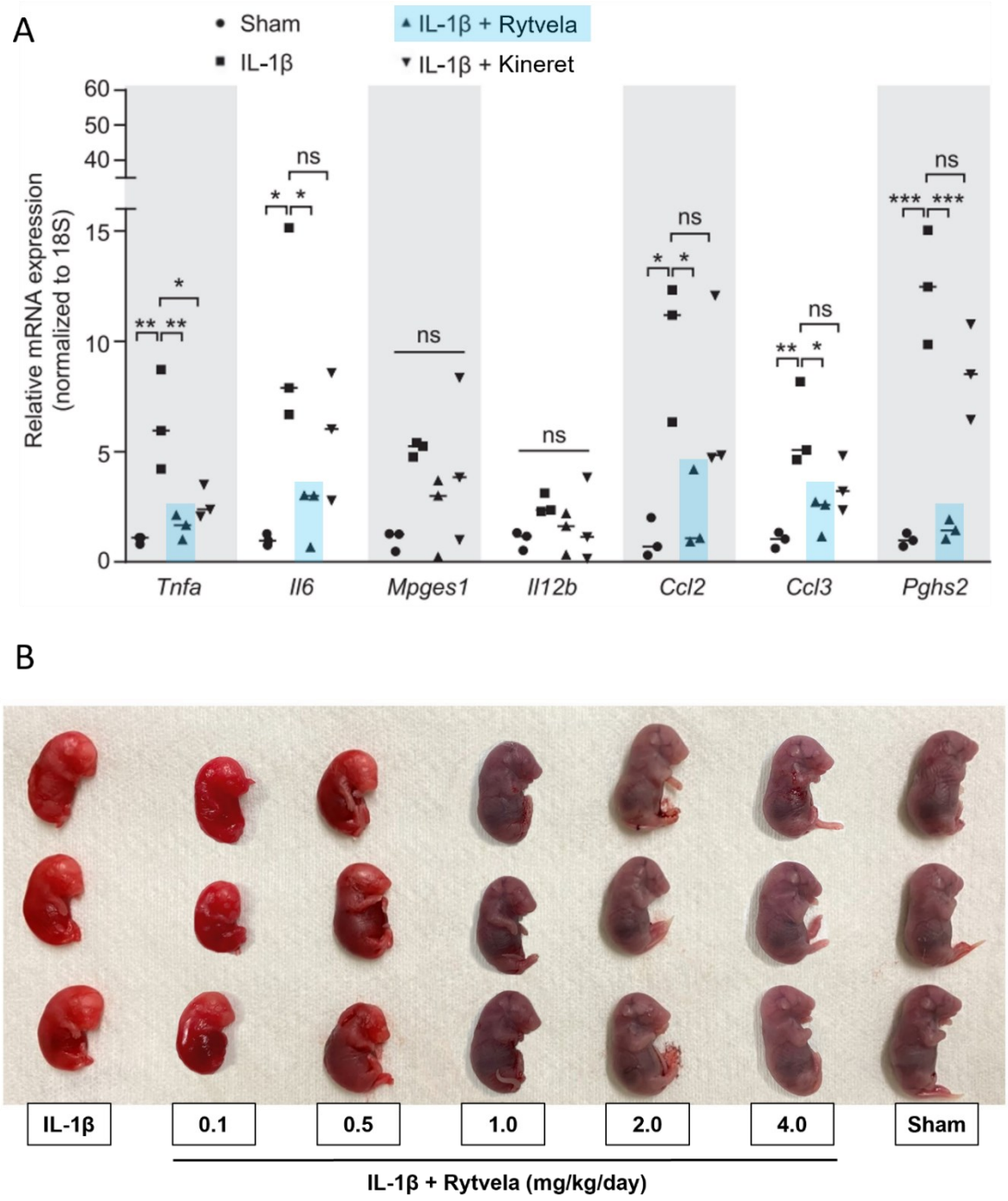

Figure 7 Treatment with Rytvela as prophylaxis prevents deleterious inflammation in the placenta and fetus of pregnant CD-1 mice exposed to IL-1β. (**A**) IL-1β-induced gene expression upregulation of proinflammatory cytokines in the placenta is prevented by Rytvela (blue bars). Placentas were collected 24 h after IL-1β injections to perform qPCR (**B**) IL-1 β -induced fetal inflammatory injury and autolysis are prevented by Rytvela in a dose-response manner. Fetuses were collected at G17. *Reproduced with the permission of Mathieu Nadeau-Vallée and Tiffany Habelrih. Data published in: Nadeau-Vallée M, et al. Antenatal Suppression of IL-1 Protects against Inflammation-Induced Fetal Injury and Improves Neonatal and Developmental Outcomes in Mice. The Journal of Immunology. 2017; Habelrih T, et al. Pharmacodynamic characterization of rytvela, a novel allosteric anti-inflammatory therapeutic, to prevent preterm birth and improve fetal and neonatal outcomes. Am J Obstet Gynecol. 2022. (25, 147)*

Furthermore, prophylaxis with Rytvela significantly improved fetal outcomes. Most fetuses from IL-1β-treated mothers had inflammatory lesions, including underdeveloped anatomy, as well as notable autolysis. Prophylaxis with Rytvela protected the fetus from the inflammatory insult in a dose-responsive manner. Maximum efficacy was achieved at a dose of 2 mg/kg/day. At this dose, the fetuses showed normal developing anatomy with no evidence of autolysis. (Figure 7B) This was consistent with improved neonatal survival, reduced inflammatory mediators in plasma, brain, lung, and intestine with associated protection of neonatal brain parenchyma, pulmonary alveolization, and intestinal mucosal integrity. Kineret had only modest effects on fetal organ protection and no beneficial effect on neonatal mortality.

No side effects were noted in the mother or the offspring.

Similar results were obtained in rodent models of preterm labour induced by bacterial-like inflammation, i.e., triggered by lipoteichoic acid (LTA) or lipopolysaccharide (LPS). (145)

These findings highlight the importance of inhibiting harmful uterine inflammation to protect the fetus and prevent preterm labor. Prevention of excessive prenatal inflammation by Rytvela prophylaxis appears to be a safe, potent, and effective therapeutic modality to protect fetal life and development.

HYPOTHESIS AND OBJECTIVES

IUGR and PTB are important worldwide socio-economic scourges. A large body of evidence supports a central role for IL-1β in both the genesis of a hostile intrauterine environment affecting fetal tissue growth and integrity, as well as in early myometrial activation leading to preterm labour. Clinical management of IUGR, PTB and their serious neonatal complications currently do not address the upstream pathological inflammatory cascade. Preclinical studies have failed to demonstrate efficacy of IL-1R large competitive antagonist Kineret in preventing PTB, but have shown a slight attenuation of harmful fetal inflammation. Our laboratory designed a small novel allosteric IL-1R antagonist Rytvela which has proven to be an effective prophylaxis to prevent PTB, noxious placental inflammation and fetal injury with better efficacy than Kineret. With these promising results, the present study aims to evaluate the effectiveness of Rytvela as a therapeutic agent, as opposed to prophylactic. In other words, we have already proved that prophylaxis with Rytvela was able to prevent the onset of a deleterious inflammatory cascade leading to PTB, and now we want to explore whether Rytvela can reverse an ongoing inflammatory reaction. This study can have important clinical ramifications, as there are no known biomarkers to predict prematurity which makes it difficult to identify patient who would benefit from prophylaxis with Rytvela. Thus, this study will explore the efficacy of Rytvela in a realistic clinical setting wherein the majority of IUGR and PTB cases are diagnosed in women with inflammatory processes already initiated.

Our hypothesis is that Rytvela, a new, small, safe and potent IL-1 antagonist, may reverse the inflammatory cascade leading to preterm labor and fetal tissue injuries primarily to the vulnerable brain, lungs and intestines.

In this sense, the objectives of the present study will be to quantify the efficacy of Rytvela in prolonging gestation of mice exposed to different inflammatory triggers and subsequently treated with Rytvela with different latencies. Also, this study aims to evaluate neonatal outcomes based on survival, growth trajectory and histological assessment of neonatal tissue integrity.

MATERIALS AND METHODS

Animals

Timed-pregnant CD-1 mice were obtained from Charles River Laboratories (Senneville, Canada) on gestational day 11 (G11). They were allowed to acclimatize for 5 days prior to experiments. Animal studies were approved by the Animal Care Committee of Sainte-Justine Hospital according to the principles of the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. The animals were kept on 12:12 hour light/dark cycle and allowed free access to standard laboratory chow and water.

Chemicals

Chemicals were purchased from the following manufacturers: Rytvela (Elim Biopharmaceuticals, Hayward, CA), recombinant human IL-1β (no. 200-01B; PeproTech, Cranbury, NJ), LPS (*Escherichia coli 0111:B4*; Sigma-Aldrich, St. Louis, MO) and LPS (*Escherichia coli 0111:B4*; Cayman Chemical Company, Ann Arbor, MI).

IL-1β–induced PTB model

IL-1β injections were performed on gestational day 16.5 (G16.5). Transposed to the timeline of human gestation (see Figure 14 as a reference for mouse vs human pregnancy timeline), the inflammatory trigger was therefore administered at the equivalent of around the 28th week of gestation (end of the 2nd trimester or beginning of the 3rd trimester). At this stage, placental growth and fetal organogenesis are completed, and a phase of accelerated fetal growth begins. (146) If the pups are born within 24h after these IL-1β injections (G16.5-G17.5), they are considered as very preterm according to the human classification of prematurity severity (see Table 1 to review classification of PTB severity in humans). If they are born between 24-48h after these IL-1β injections (G17.5-18.5), they most likely fit in the moderate to late PTB category according to human classification. At G19, pups are considered to be at term.

Pregnant CD-1 mice were anesthetized with an isoflurane mask for the complete duration of the procedure. A laparotomy was performed (medial incision in the lower abdominal wall). The exposed uterine horns were injected with 1 μg of IL-1β between two fetal membranes with special care not to enter the amniotic cavity (intrauterine [i.u.] administration). This model intends to simulate a local sterile inflammatory response. Indeed, gestational inflammation can be due to microorganisms (e.g., bacteria, parasites, or viruses) or to other mechanisms of disease in which cellular stress induces a release of damage-associated molecular patterns (DAMPs) which activate the innate immune system and the production of pro-inflammatory cytokines like IL-1β, among others. The term "sterile inflammation" refers to an inflammatory process in which microorganisms cannot be detected. (89) Since IL-1β is an endogenous molecule (as opposed to exogenous bacterial component triggers of inflammation such as the widely used LPS), we used this model as a simulation of sterile inflammation.

The abdominal muscle layer was sutured, and the skin closed with clips. Rytvela (1.0 mg/kg/12h), or a vehicle (saline water) was injected in the neck (subcutaneous [s.c.] administrations) of the pregnant CD-1 mice at different time points (0.5, 2.0, 4.0 or 6.0 hours) after IL-1β injections across the experimental groups (n = 5-14 dams). Peak inflammation has been reported to occur around 2 hours after stimulation with inflammatory triggers, including LPS. (148, 149, 150) Systemic inflammation induced with LPS showed an elevation of TNF-α levels by approximately 4500% compared to controls after only 2 h in a mouse model, which highlights the rapid and strong inflammatory response in mice. (151) IL-1β and IL-6 were also found to be significantly increased 2 hours after LPS injections in mice. (148) In a mouse model of preterm labor induced by intrauterine LPS injection, measurements at 3h post-LPS of myometrial and circulating levels of chemokines and cytokine were significantly elevated. (152) Hence, Rytvela's efficacy was tested at time points between 0.5 to 6 h after inducing inflammation with IL-1 or LPS (encompassing the peak inflammation reported in literature) in an attempt to evaluate reduction in maternal inflammatory processes and reduction in pro-inflammatory cytokine transfer to gestational tissues and fetuses.

The dosage of Rytvela was chosen according to a dose-response study conducted in our laboratory showing that maximum efficacy was achieved at a dose of 2 mg/kg/day when Rytvela was administered prophylactically (see Figure 7B). (147)

Rytvela was administered twice daily until G18.5. The mice pregnancies were assessed every day. Newborn survival and litter size were assessed at birth. Pups (four per litter) remained with their dam and were killed on post-term day (PT) 7 for further histological analysis. See Figure 8 for a diagram of the experimental design.

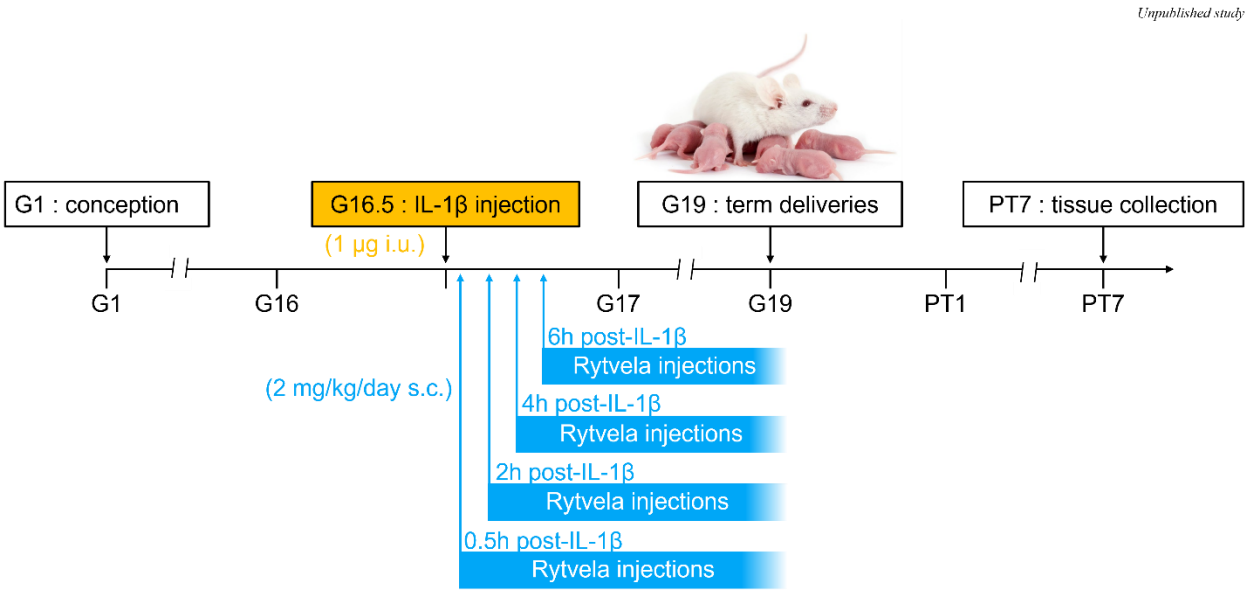

Figure 8 Experimental design of IL-1β–induced PTB model.

LPS-induced PTB and IUGR model

Pregnant CD-1 mice were injected with 10 µg of LPS (*Escherichia coli 0111:B4*; Sigma-Aldrich, St. Louis, MO) intraperitoneally (i.p.) on G16.5. This model intends to mimic a systemic gram-negative infection, as studies have shown that bacterial endotoxin LPS induced a significant increase in proinflammatory and prolabour mediators in gestational tissues, including IL-1. (79, 153) Rytvela (1.0 mg/kg/12h), or vehicle (saline water) was injected in the neck (subcutaneous [s.c.] administrations) of pregnant CD-1 mice at different time points (0.5, 2.0, 4.0 or 6.0 hours) after LPS injections in the different experimental groups (n = 5-17 dams). Rytvela was administered twice daily until G18.5. The mice pregnancies were assessed every day. Newborn

survival and litter size were assessed at birth. Pups (four per litter) remained with their dam and were killed on post-term day (PT) 7 for further histological analysis. Pups body and brain weight were assessed at PT7. See Figure 9 for a diagram of the experimental design.

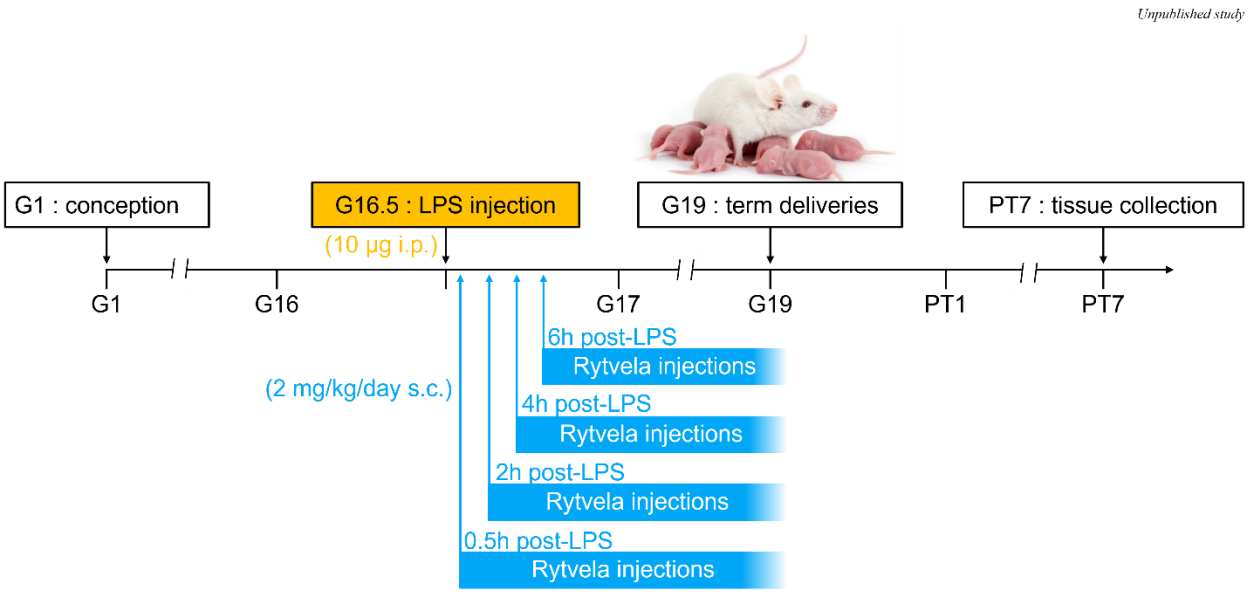

Figure 9 Experimental design of LPS–induced PTB model.

PTB and IUGR model induced by two-time LPS exposure

Based on in vivo dose-response and "time-response" experiments that lead to PTB, IUGR, and neonatal tissue damage in a reproducible manner, our colleague Dr Xin Hou developed a model with two-time exposition to LPS during late pregnancy in mice (which corresponds to the end of the 2nd trimester in human pregnancy): 4 µg of LPS (*Escherichia coli 0111:B4*; Cayman Chemical Company, Ann Arbor, MI) were administered i.p. at G16 followed by a second administration of 6 µg of LPS i.p. on G17. This model intends to mimic a more chronic or subacute state of inflammation in advanced pregnancy. As the second trimester (from 14 to 28 weeks in human) occurs approximately from G14-G17 in mice, the one-day inflammatory exposition interval in mice would approximately correspond to a 4-weeks interval in human (see Figure 14 as a reference for mice vs human pregnancy timeline).

Rytvela (1.0 mg/kg/12h), or a vehicle (saline water) was injected in the neck (subcutaneous [s.c.] administrations) of pregnant CD-1 mice at different time points (0.5, 2.0 or 6.0 hours) after the

second LPS injections in the different experimental groups (n = 8-11 dams). Rytvela was administered twice daily until G18.5. The mice pregnancies were assessed every day. Newborn survival and weight were assessed at birth. Pups (four per litter) remained with their dam and were killed on post-term day (PT) 7 for further histological analysis. Pups weight and length were assessed at PT7. See Figure 10 for a diagram of the experimental design.

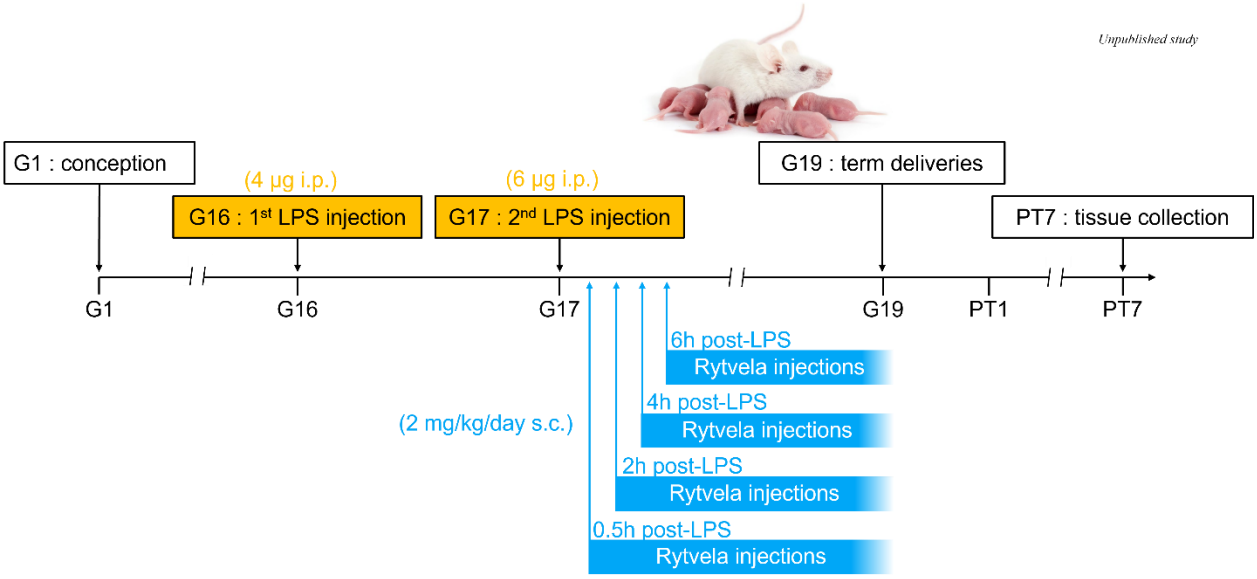

Figure 10 Experimental design of PTB and IUGR model induced by two-time LPS exposure.

Tissue collection and fixation

Histological analyses of the fetal lungs, intestines and brain were performed to assess the growth and the integrity of fetal tissues. Tissues were obtained from 2 pups per dam from 6 to 8 dams per group for each model (total of 180 dams and 360 pups) with the help of Dr Xin Hou. At PT7, the pups were anesthetized with isoflurane. They were perfused intracardially first with saline solution containing heparin, and second with 10% formalin (Fisher Scientific). They were intubated intratracheally, and lungs were perfused with 10% formalin (Fisher Scientific). The pups' heads were severed before their brain, intestines, and lungs were collected. The cranium was opened following the sagittal suture and the brain was carefully extracted and weighted. Then, the ileum (±5 cm long just above the cecum) was excised and irrigated with a protease inhibitor solution (SIGMAFAST Protease Inhibitor Tablet, S8820, Sigma-Aldrich, St. Louis, MO) to

remove any feces and to ensure the absence of post-mortem degradation of the tissue by protease. Lungs, filled with formalin, were excised in bloc with the heart (to facilitate orientation of the sections). All tissues were fixed in 10% formalin for at least 24 h. Lungs and intestines were subsequently transferred to phosphate buffered saline (PBS) at 4˚C. Brains were saturated overnight at 4°C in a 30% sucrose solution and subsequently transferred to PBS at 4˚C. Lungs and intestines were embedded with paraffin before performing microtome cuts. Brains were frozen in a water-soluble embedding medium (Frozen Section Compound, FSC 22 Clear) using dry ice and stored at -80°C before performing cryostat cuts.

Lung, intestine, and brain histology

Five micrometer–thick coronal sections were performed on paraffin-embedded lungs. Five micrometer–thick longitudinal sections were performed on paraffin-embedded intestines. The sections were stained with H&E. The cuts and staining were performed by the CHU Sainte-Justine Pathology Department - this collaboration was made possible with the help of Dre Natasha Patey.

Thirty micrometer–thick coronal sections were performed on brains with the Cryostat (Leica) with the help of Dre Isabelle Lahaie and Dre Irene Londono. The brain vessels were labeled with a FITC-conjugated lectin (1:200; FL-1101; Vector Laboratories, Brockville, ON, Canada). The nuclei were stained with DAPI (1:5000; Invitrogen). Brains were mounted on microscope slides (Bio Nuclear Diagnostics Inc., Toronto, ON) under cover slips with Fluoro-Gel® (Electron Microscopy Sciences, Hatfield, PA) as the mounting media.

Images of all tissue sections were acquired using a 10X objective with a high-resolution slide scanner (Axio Scan, Zeiss, Jena, Germany).

Histological analysis

Histological analyses were performed using the Zen3 software with the guidance of Dre Elke Küster-Schöck. The high-resolution scanned tissue sections were carefully analysed, and multiple regions of interest (ROI) were selected from each tissue section for semi-quantitative analysis.

For lungs, the analysis was carried out by a script written in Python specifically designed by our collaborator Allan Reuben. This script measured the average alveolar area and alveolar count

from the mean of two to four regions of interest (ROI) of 1 mm^2 in each tissue section analyzed. The script was useful to analyse large samples and to limit analysis bias.

For intestines, ileum diameter and villus height (from the basal layer of the submucosa to the ending of the villus) were measured manually. The average length was calculated based on over 20 measures per tissue sections.

For brains, vascular density was calculated from 2-4 ROI of 750x750 microns in the cortex area of each brain tissue section. We used ImageJ software (analysis protocol by Dre Elke Küster-Schöck) to create a binary image from our ROI, and to determine the percentage of vessel coverage, allowing for semi-quantitative comparisons of the vascular density.

Statistical analysis

All data was analyzed using GraphPad Prism version 9.0 software (GraphPad Software, San Diego, CA). A one-way analysis of variance (ANOVA) was employed for most of the data with a Dunnett multiple comparison method to compare data to a single control (the inflammatory group of IL-1β or LPS). A Chi-square test was used to compare the binary variable (including preterm birth rates and survival rates). A p value of < 0.05 was considered statistically significant. Data is presented as means ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

1. Rytvela prevents adverse obstetrical and perinatal outcomes when administered after inflammation onset

Our previous data suggested that Rytvela could prevent the inflammatory cascade leading to PTB and neonatal morbidity and mortality when given as a prophylaxis. To study the efficacy of Rytvela, as a therapeutic agent (as opposed to prophylactic), in stopping the inflammatory cascade leading to IUGR, PTB and neonatal mortality, we used 3 different animal models of inflammation-induced PTB. We administered either 1 dose of intrauterine IL-1β, either 1 dose of intraperitoneal LPS, or either 2 doses of intraperitoneal LPS in late gestation (G16-G17, term deliveries being at G19). The first model is meant as a sterile model of uterine inflammation. The two latter are clinically relevant model triggered by bacterial products; an important upstream cause of uterine inflammation associated with poor neonatal outcomes. Besides, LPS is generally widely used in research for its ability to generate a significant immune response. We started a treatment with maternal subcutaneous Rytvela at different time points (from 0.5h to 6h post-inflammatory triggers) until delivery (twice daily at the dosage of 2.0 mg/kg/day) to evaluate the potential of Rytvela as a therapeutic agent. We carefully assessed time of delivery and newborn survival.

1.1 Rytvela prevents PTB and neonatal mortality in all 3 models of inflammation-induced PTB

In the IL-1β model, the 57% rate of PTB in the inflammation-exposed group was decreased to 0% with the administration of Rytvela 0.5h post-IL-1β (Figure 11B). All litters treated with Rytvela 0.5h post-IL-1β survived, compared to only 43% of survival in the untreated litter exposed to IL-1β (Figure 11C). Among the surviving litters, 66% of the IL-1β exposed group had 7 pups or under, whereas among all Rytvela treated groups (from 0.5 to 6.0 hours post- IL-1β) 83% of litters had more than 9 pups (Figure 11D). Knowing that the minimal litter size of our healthy control groups (Sham) in all experiments was of 8 pups, we can consider that a significant proportion of the

surviving litters antenatally exposed to IL-1β were abnormally small, with the smallest being of 3 pups (i.e., part of the litter exposed to antenatal IL-1β did not survive the inflammatory environment). On the other hand, Rytvela saved most of the pups, leading to larger litters. Rytvela is statistically efficient 0.5h post-IL-1β in preventing PTB and neonatal death (Figure 11B, 11C). A tendency of improvement of these gestational and perinatal outcomes is observed at 2h and 4h post-IL-1β, but larger cohorts might be necessary to confirm the efficacy of Rytvela when administered after a prolonged delay (greater than 0.5h post- IL-1β).

In both LPS models, the significant PTB rates of up to 91% of litters decreased more than 2-fold with Rytvela administered 0.5h post-LPS (Figure 12B, 13B). Litter survival rate almost doubled with Rytvela treatment at 0.5h post-LPS (Figure 12C, 13C). Again, a tendency of improvement of these gestational and perinatal outcomes is observed at 2h and 4h post-LPS. The magnitude of improvement in these outcomes appears to be time dependent – the earlier Rytvela is administered, the more perinatal outcomes improve. There may be beneficial effects even with delayed administration (e.g., there was a near 2-fold decrease in PTB rate with Rytvela administered 4h post-LPS (Figure 12B)), but larger cohorts would be necessary to confirm the statistically significant efficacy of Rytvela when administered after a prolonged delay. Also, delayed treatment may be more efficient with a higher dosage of Rytvela.

As mentioned above, in the present study, the doses were chosen according to a dose-response study wherein maximum effect was obtained at a dosage of 2 mg/kg/day in which Rytvela was administered in prophylaxis (147). A therapeutic dose of a given molecule (e.g., anticoagulant) must usually be higher than a prophylactic dose. As Rytvela was administered therapeutically in this study, we can hypothesize that higher doses (> 2 mg/kg/day) would have been more efficient especially in delayed treatments. Considering the peak inflammation reported in literature around 2-3h after initial insult, prompt administration of Rytvela (at 0.5h) is efficient at a prophylactic dosage (2 mg/kg/day), but as we approach the peak inflammation a prophylactic dosage of Rytvela may be insufficient for optimal management. In other words, in case of tardive administration, Rytvela would likely be more effective in greater dosage as such would be required to adequately reverse the inflammatory peak and prevent PTB.

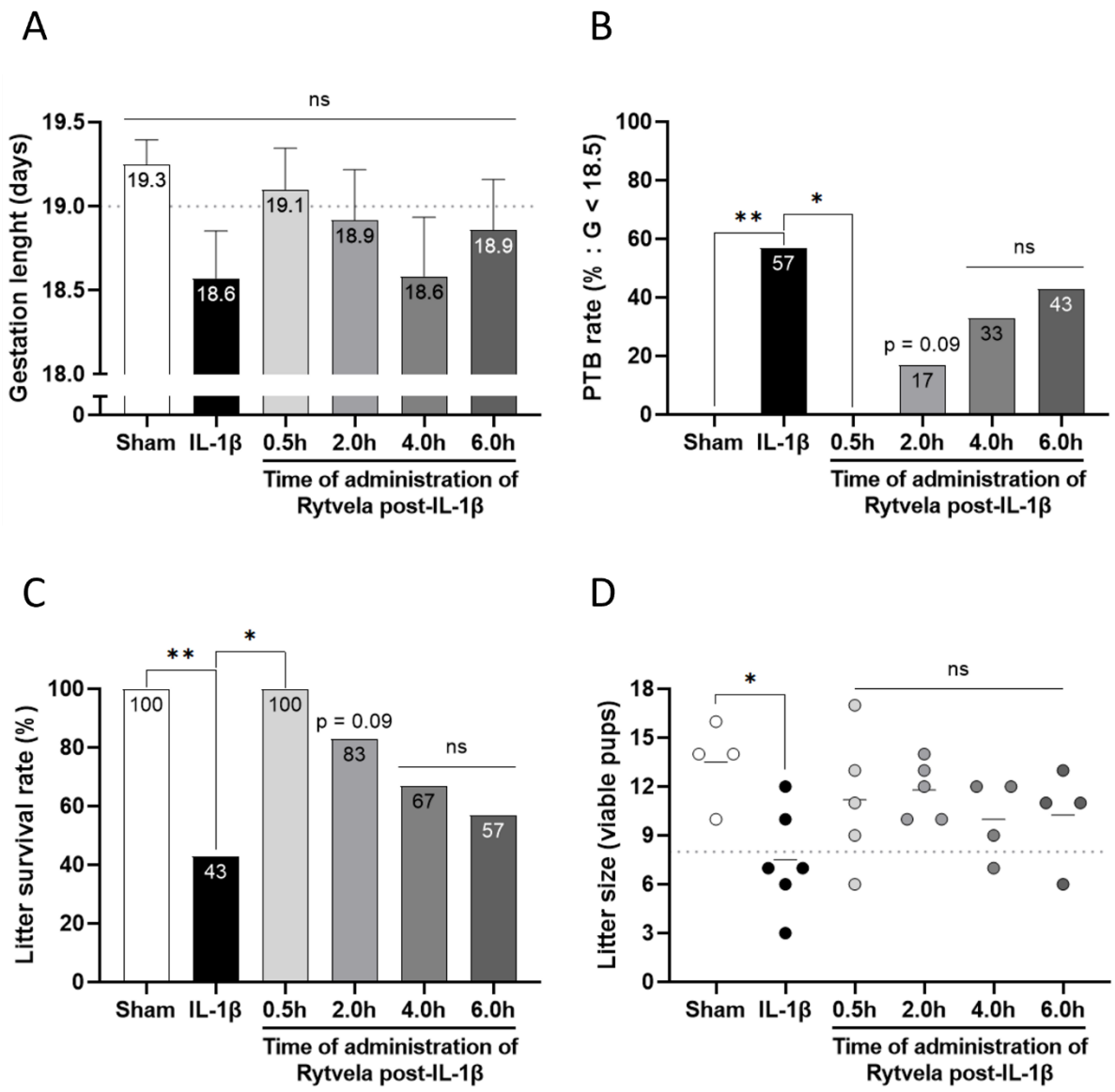

Figure 11 IL-1β-induced PTB and offspring mortality are prevented by administration of Rytvela after the inflammatory insult. (**A**) Mean gestation length compared to a normal gestation length of 19 days. (**B**) PTB rate: percentage of litters delivered before 18.5 days of gestation. (**C**) Percentage of litters with surviving newborns. (**D**) Number of viable pups per litter compared to the minimal litter size of 8 pups in the Sham group of all experiments. *n* = 5-14 dams per group for A-C, and 4-6 dams per group for D. Values are presented as means ± SEM. **p* < 0.05, ***p* < 0.01 by one-way ANOVA with Dunnett post-analysis for A, D and by Chi-square analysis for B, C.

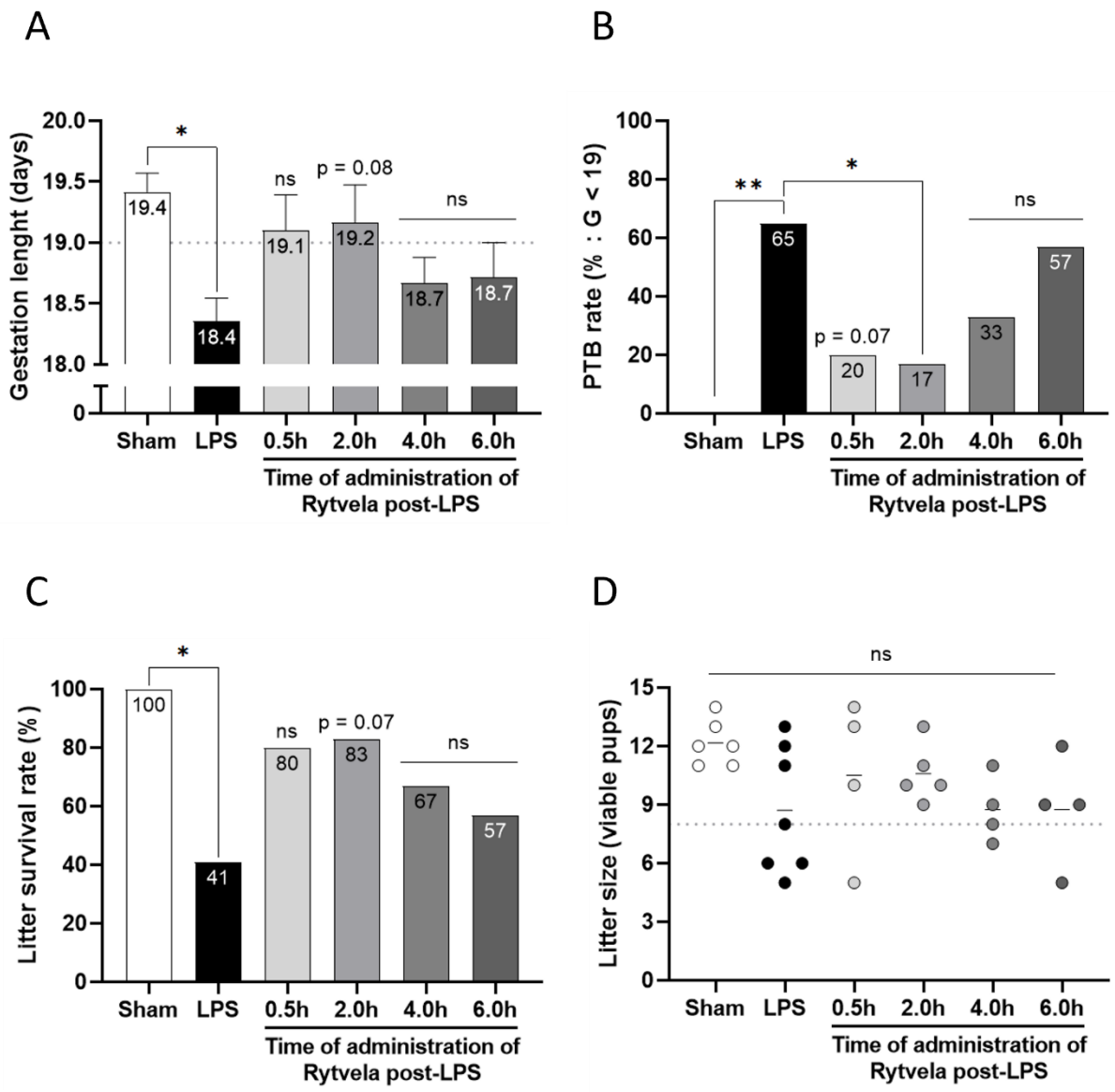

Figure 12 LPS-induced PTB and offspring mortality are prevented by administration of Rytvela after the inflammatory insult. (**A**) Mean gestation length compared to a normal gestation length of 19 days. (**B**) PTB rate: percentage of litters delivered before 19 days of gestation. (**C**) Percentage of litters with surviving newborns. (**D**) Number of viable pups per litter compared to the minimal litter size of 8 pups in the Sham group of all experiments. *n* = 5-17 dams per group for A-C, and 4-7 dams per group for D. Values are presented as means ± SEM. **p* < 0.05, ***p* < 0.01 by one-way ANOVA with Dunnett post-analysis for A, D and by Chi-square analysis for B, C.

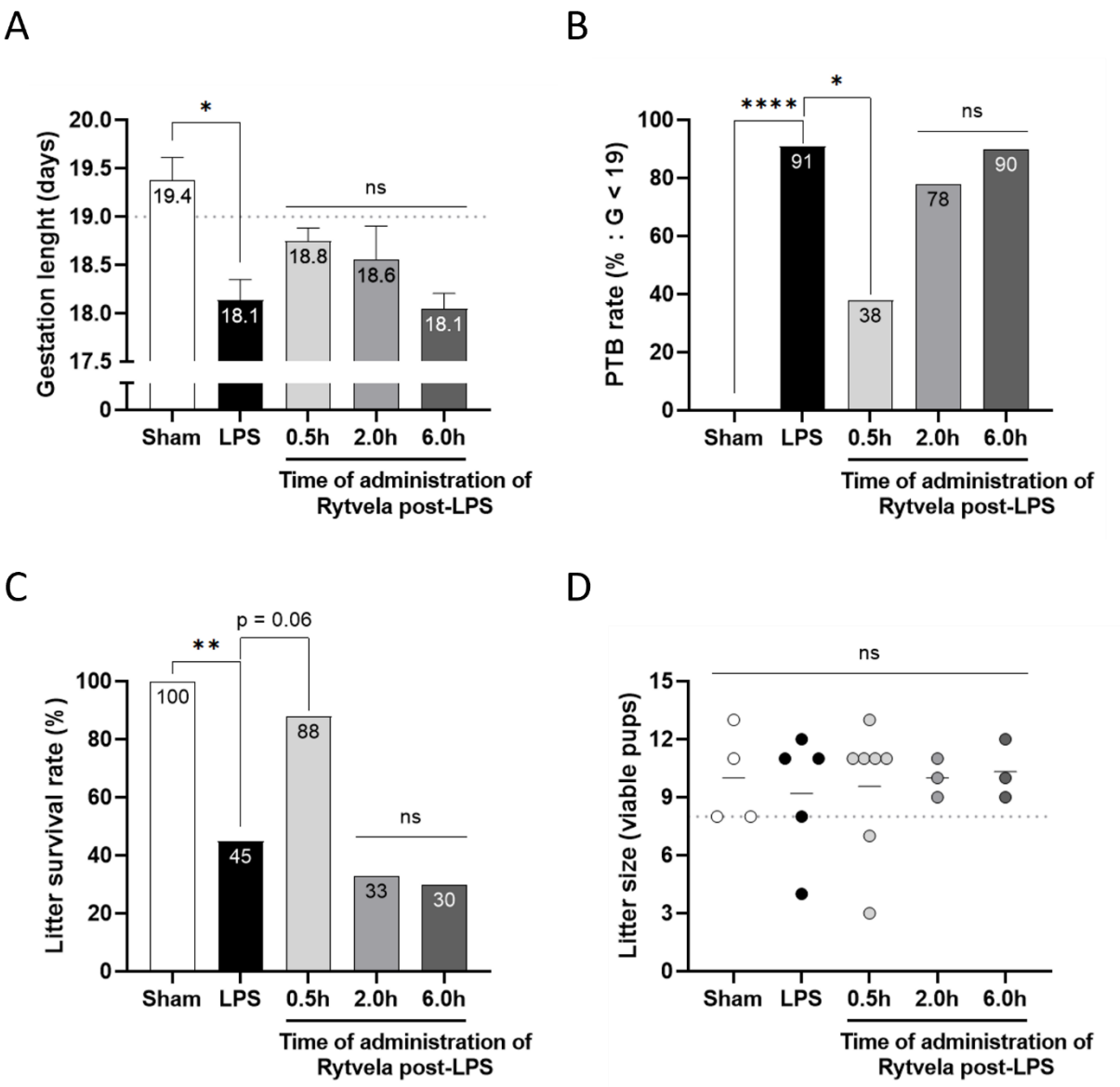

Figure 13 PTB and neonatal mortality induced by antenatal two-time exposure to LPS are prevented with the administration of Rytvela after inflammatory onset. (**A**) Mean gestation length compared to a normal gestation length of 19 days. (**B**) Percentage of preterm birth (PTB) in each group: litters delivered before 19 days of gestation. (**C**) Percentage of litters with surviving newborns. (**D**) Number of viable pups per litter compared to the minimal litter size of 8 pups in the Sham group. *n* = 4-11 dams per group for A-C, and 3-7 dams per group for D. Values are presented as means ± SEM. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001 by one-way ANOVA with Dunnett post-analysis for A, D and by Chi-square analysis for B, C.

In order to apply these results to the human clinical context, the mouse pregnancy timeline should be contextualized against the human equivalent. Mice have a relatively short length of gestation (19 days) compared to humans (9 months or 40 weeks or 280 days). (154) Mice have a total gestation length approximately 14 times shorter than humans. Half a day for the pregnant mouse corresponds to approximately one week in humans. (146) As such, we could tentatively extrapolate that treating an acute inflammatory process in the pregnant mouse in a 30 min delay would represent a 7h delay on the human scale, whereas a 6h delay for mice would correspond to 3.5-day delay for humans. Indeed, everything goes faster in mice compared to humans including reproduction (menstrual cycle, fetal development, parturition), but also generally metabolism (155), maturational rate (up to 150 times faster), and senescence (156).

Moreover, in mice, the trimesters are not proportionally divided like the human trimesters. The first trimester's milestones (implantation, placentogenesis, organogenesis) are reached after more than half of the total gestation length, typically around 14 days. The second and third trimesters (mainly for fetal growth) last only about 5 days. The fetal growth is mostly accelerated in the last 2 days of the mouse pregnancy which corresponds for humans to the third trimester which is about 12 weeks or 84 days (Figure 14). All this considered, working with pregnant mice models, particularly in the second and third trimester, implies that physiological processes and fetal development are greatly accelerated compared to humans.

The prolonging of gestation by 0.5 to 0.8 days in our models with the treatment of Rytvela could approximately correspond to a 1-week to 11-day prolonging in humans if compared according to the respective gestation length of mice and humans. (146) Considering the non-proportional trimesters of mice as opposed to humans (Figure 14), a prolongation of gestation of 0.5 days in the third trimester in mice actually represents a 25% increase in the trimester length, which could potentially correspond to more than 3 weeks of human fetal development and growth.

The exact correspondence in humans of a 0.5-day extension in mice gestation is difficult to assess precisely. We can imagine that it represents at least one or several weeks as discussed above, and that Rytvela could therefore be at least non-inferior and likely superior to the present first-line clinical candidate for tocolysis Nifedipine (ineffective in delaying labour beyond 7 days). Also, as

already mentioned, Nifedipine inhibits myometrial contractions by blocking the intracellular calcium influx, but it does not appropriately address the upstream inflammatory cascade that keeps the fetus in a hostile inflammatory environment. Thus, Rytvela is a more promising molecule than Nifedipine in terms of perinatal outcomes.

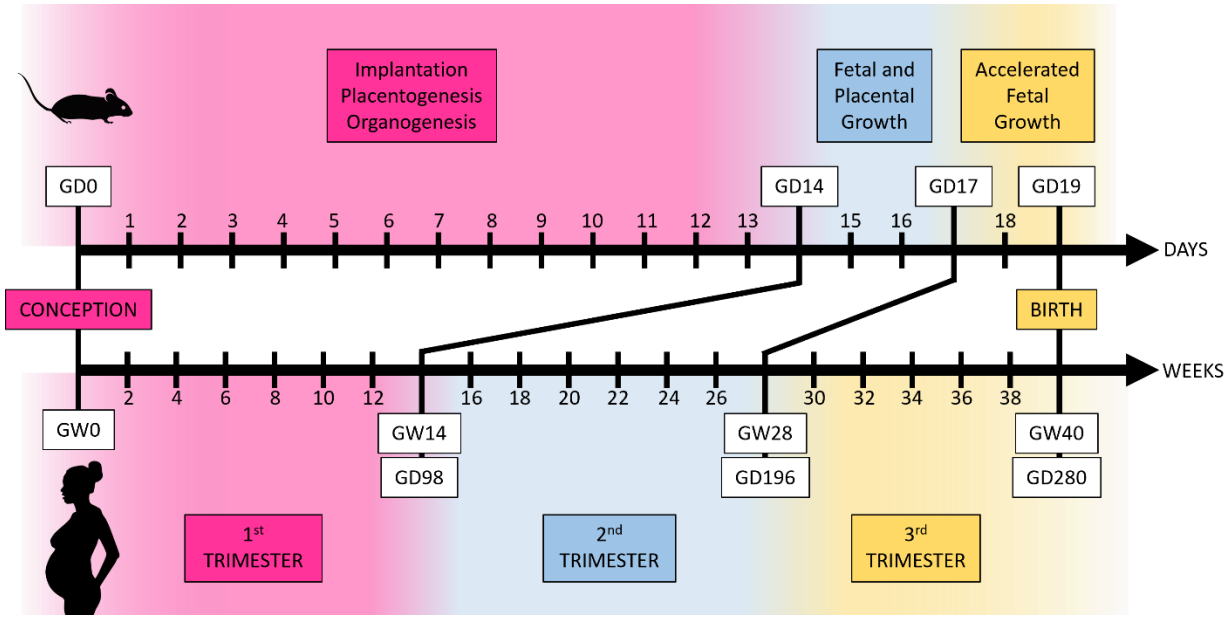

Figure 14 Timeline of mouse gestation and analogous trimester demarcations in human pregnancy based on Theiller stages of mouse development and Carnegie stages of human development. GD, gestation day; GW, gestation week. *Conception of this figure was inspired from: Blum JL, et al. Exposure to Ambient Particulate Matter during Specific Gestational Periods Produces Adverse Obstetric Consequences in Mice. Environ Health Perspect. 2017. (146, 157, 158)*

As discussed earlier in the introduction, inflammation is an essential physiological defense mechanism enabling protection against infectious and non-infectious insults. It is also essential for gestational tissue reparation and transformation throughout a physiological pregnancy. However, inflammatory processes can become pathological if they are triggered at the wrong time, in the wrong place, at the wrong intensity, especially in the context of the delicate balance of pregnancy. Many inflammatory triggers can significantly impact pregnancy outcomes from chronic auto-immune disease or toxic substances exposure to acute traumatic or infectious insults. These triggers can prematurely switch the balance from uterine quiescence to uterine activation and induce preterm labour and birth. PTB has been the leading cause of infant mortality in the world for several decades, but there is still no effective treatment. Our mice models recreate the setting of excessive sterile (with IL-1β injections) or infectious (with LPS injections) inflammation in late pregnancy resulting in high rates of preterm birth. Prophylactic treatment of these pregnant mice with our small peptide Rytvela has proven to be very promising in previous studies. In this study, we are now able to demonstrate that Rytvela is efficient not only as a prophylactic, but also as therapeutic agent. Rytvela significantly reduces the number of preterm deliveries and significantly improves fetal survival when administered 0.5h after intense acute inflammatory triggers (with 1 µg of IL-1β i.u. or 10 µg of LPS i.p.), as well as after milder subacute inflammatory triggers (in the two-time LPS exposure model with 4 µg of LPS i.p. followed by 6 µg 24h later). The latter could possibly imply that delayed treatment with Rytvela may be effective in the context of milder subacute immune activation (Rytvela being effective more than 24h after the first inflammatory trigger in the two-time LPS exposure model), whereas severe acute inflammatory processes require more prompt attention. More research on the effectiveness of delayed therapy with Rytvela with higher dosage would be interesting.

These results most importantly highlight the central role of IL-1, not only in the onset, but also in the maintenance of the inflammatory cascade leading to PTB, with the production of more proinflammatory cytokine and recruitment of leukocytes, and changes in the gestational tissues leading to preterm labour. With the prophylactic administration, we showed we could prevent the onset of this inflammatory cascade. With the therapeutic administration, we now show that we can reverse ongoing inflammatory processes. This has very interesting clinical ramifications.

To date, there are no measurable biomarkers to predict prematurity, making it difficult to identify patients who would benefit from Rytvela prophylaxis. In this sense, a hypothetical case of preterm labour in our medical context might likely look as follows. A pregnant woman in preterm labour may present to the hospital with spontaneous contractions or preterm premature rupture of membranes (PPROM) for example, and thus with an acute sterile and/or infectious inflammatory process in progress. Clinicians will most likely hydrate her with IV fluids, monitor uterine contractions and the fetal heart, administer tocolytics to attempt to reduce uterine contractility, give her betamethasone for maturation of the baby's lungs, and transfer her to a tertiary care center for optimal management of the preterm newborn by qualified neonatologists (and give her antibiotics in case of PPROM to prevent chorioamnionitis). All this for an average gain of only about 48 hours of gestation (or maybe 7 days with Nifedipine if we are lucky), which is far below the desired effect of pregnancy prolongation. The result is a very high proportion of premature deliveries despite our interventions with high rates of neonatal complications. Obstetricians need much more potent agents to slow down ongoing preterm labour. This explains why the efficacy of Rytvela in suppressing ongoing acute inflammatory processes may be crucial in the eventual management of prematurity in humans.

1.2 Rytvela prevents FGR in LPS-induced PTB models

In our LPS exposed groups, a serious impact on the fetus and newborn growth was noted (Figure 15A, 16A), suggesting that short-term antenatal exposure to inflammation is sufficient to affect late prenatal and postnatal growth in our mice model. Treatment with Rytvela after inflammation onset preserved fetal and neonatal growth and brain development (Figure 15B, 15C, 16A, 16B). In the single LPS exposure model, Rytvela significantly protected the pups from FGR when administered up to 4h post-LPS (Figure 15). In the two-time LPS exposure model, Rytvela significantly preserved pups' weight and length when administered 0.5h post-LPS (Figure 16).

As discussed earlier in the introduction, maternal inflammation can reach the fetus as pro-inflammatory cytokine can cross the placenta and create a toxic inflammatory environment for the developing fetus, resulting in impaired fetal growth. Animal models of maternal systemic inflammation with repeated exposure to LPS in the third trimester resulting in marked FGR are described in literature. There is also ample supportive evidence that, regardless of the exact etiology of IUGR, a significant proportion of cases involve inflammatory processes in their pathophysiology, as reflected by the increased presence of proinflammatory mediators on both the maternal and fetal sides in IUGR cases. Currently, except for prophylaxis with low-dose aspirin, whose real effectiveness seems to be limited to specific cases, there is no pharmacological treatment for FGR. Yet, like prematurity, FGR has devastating impacts on newborns with high rates of serious postnatal complications such as bronchopulmonary dysplasia, necrotizing enterocolitis, cerebral palsy and other neurodevelopmental disorders. Our mice models recreate the setting of excessive maternal inflammation in late pregnancy resulting in marked FGR. In this study, we report for the first time the efficacy of Rytvela in preventing this FGR.

This further support the capital role of IL-1 in the pathophysiology of a wide range of conditions, including adverse obstetrical outcomes such as IUGR and PTB. IL-1 is at the apex of the production of many proinflammatory mediators that cross the placenta and impair fetal development and growth.

The current clinical approach for IUGR is mostly based on induced preterm delivery with the idea that the natural incubator (i.e., the intrauterine environment) is failing and that the fetus may be

better supported in an artificial incubator in the neonatal intensive care unit. Our results could represent a real paradigm shift in the management of IUGR. What if we could significantly improve the natural incubator by reducing pathological fetomaternal inflammation with a safe and potent anti-inflammatory agent like Rytvela? This deserves further exploration.

In short, Rytvela exhibits a convincing positive impact on gestation length, and offspring's survival and growth when administered as a therapeutic agent (and not just as a prophylaxis). We need further analysis with wider samples to confirm the efficacy of a delayed treatment with Rytvela in preventing PTB and neonatal mortality. However, data presented in this section (Figure 11-16) show an impressive and statistically significant improvement in adverse gestational and perinatal outcomes with Rytvela treatment at 0.5h post-inflammatory triggers.

With these robust results, we decided to take a closer look at the impact of prenatal exposure to inflammation treated with Rytvela on fetal tissue integrity.

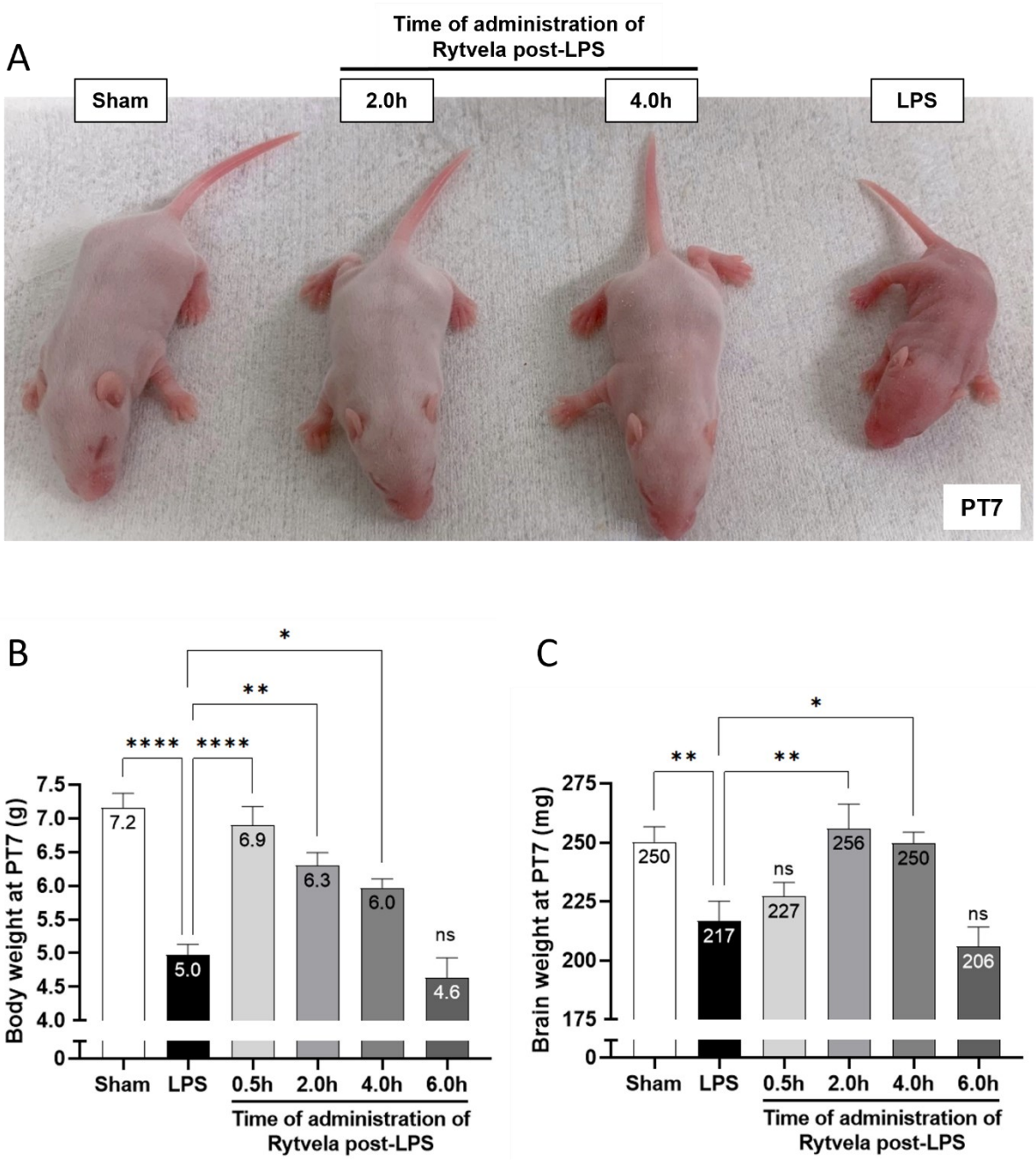

Figure 15 LPS-induced FGR is prevented by administration of Rytvela after inflammatory onset. (**A**) Representative picture of pups at 1-week post-term (PT7). (**B**) Pups mean body weight at 1 week of life, on post-term day 7 (PT7). (**C**) Pups mean brain weight on post-term day 7 (PT7). *n* = 7-18 pups per group for B, and 8-14 pups per group for C. Values are presented as means ± SEM. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001 by one-way ANOVA with Dunnett post-analysis.

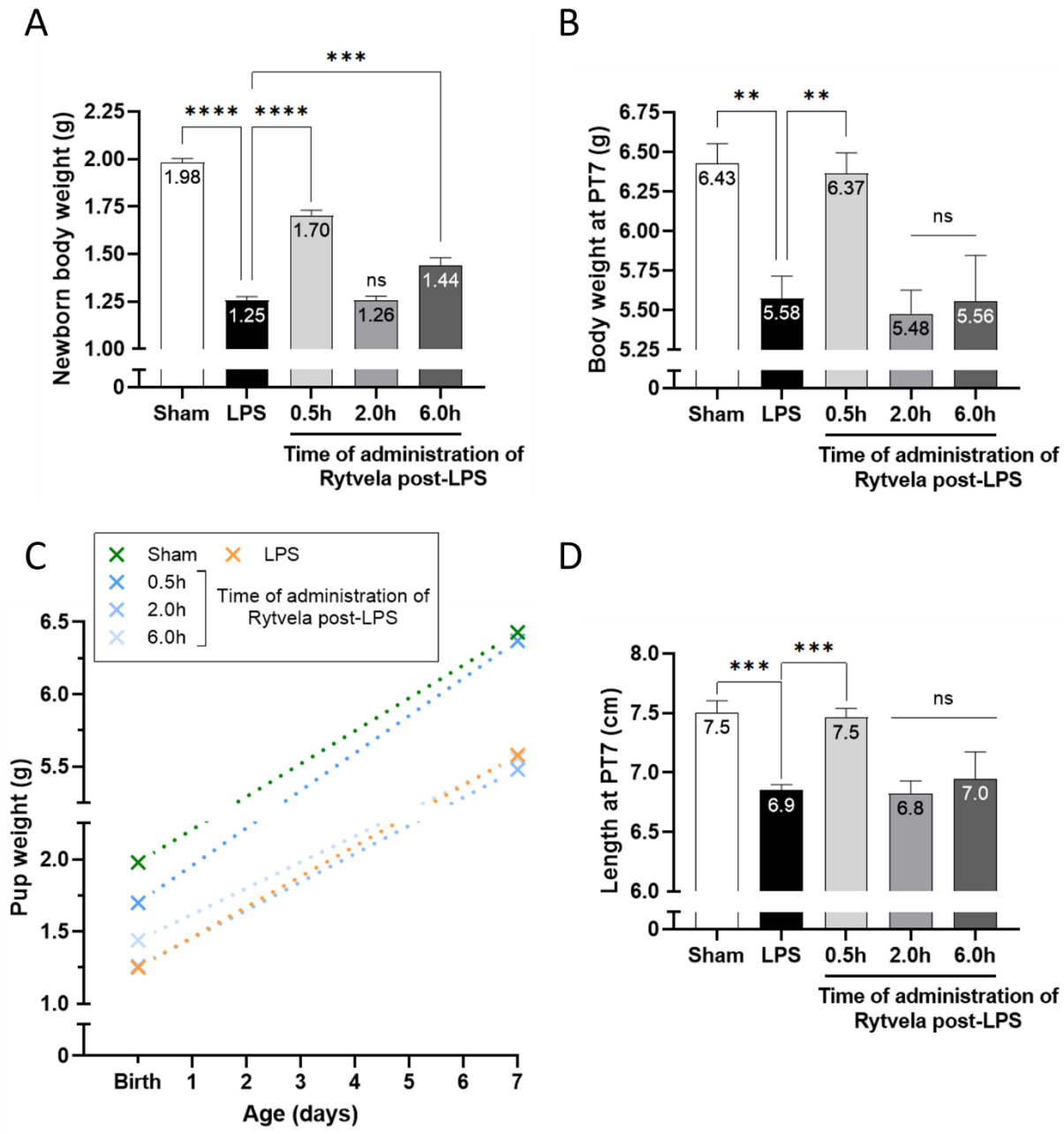

Figure 16 FGR induced by antenatal two-time exposure to LPS is prevented with the administration of Rytvela after inflammatory onset. (**A**) Pups mean body weight at birth. (**B**) Pups mean body weight at 1 week of life (PT7). (**C**) Growth trajectory estimates based on each group mean weight at birth and mean weight at 1 week old. (**D**) Pups mean craniocaudal length (measured from the muzzle to the tip of the tail) at 1 week old. *n* = 23-74 pups per group for A, 8-34 pups per group for B, D, and 12-74 pups per group for C. Values are presented as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by one-way ANOVA with Dunnett post-analysis.

2. Rytvela prevents morphological alterations in lungs, intestines, and brain when administered after inflammation onset

Given the positive impact of Rytvela on survival and growth of offspring, we wanted to further characterize its protective effect on newborn most vulnerable organs with histological analysis. Several neonatal tissues including lungs, intestines, and brain are particularly prone to inflammatory damages that begin before birth and are exacerbated by premature exposition to post-natal environment. In the context of IUGR and PTB, the prenatal intrauterine environment becomes hostile as proinflammatory cytokines from the mother cross the placenta and penetrate the fetal circulation and amniotic fluid. Figuratively speaking, the fetus is bathed in an inflammatory hot soup. The postnatal environment on the other hand is characterized by hyperoxia, mechanical stress, and pathogens, among others, which are all potential threat to the fragile immature infant. Excessive inflammation, triggered by various antenatal and postnatal stressors, is a common upstream pathway observed in severe perinatal diseases like bronchopulmonary dysplasia, necrotizing enterocolitis, and neonatal encephalopathy. To determine whether treatment with Rytvela could reverse the antenatal systemic inflammatory response associated with abnormalities in fetal and neonatal organ development (as is observed in humans), we collected the pup's lungs, intestines, and brain on post-term day (PT) 7 for histological analysis.

2.1 Rytvela protects fetal lungs in all 3 models of antenatal inflammation

In utero exposure to IL-1β induced, as expected, marked anomalies in pups' lungs parenchymal architecture with disrupted alveolarization. Treatment with Rytvela up to 4h post-IL-1β protected the pups' lungs which displayed a normal pulmonary parenchyma similar to our Sham group. (Figure 17A). Semiquantitative analysis of lung morphology revealed that the significant decrease in alveolar count and increase in alveolar size in pups exposed to IL-1β were prevented by Rytvela treatment up to 4h after the inflammatory insult. (Figure 17B, 17C).

During normal gestation, amniotic fluid is physiologically inhaled by the fetus. In an adverse obstetrical setting such as IUGR and PTL, the amniotic fluid, containing proinflammatory cytokines

such as IL-1β, actually acts as an additional vector of inflammation to the fetal lungs. This inflammation is thought to play an important role in the pathogenesis of BPD, by directly inhibiting alveolar and microvascular development in the fetal lungs.

Our mouse model recreates the context of excessive intra-amniotic inflammation, which results in an atypical lung parenchyma in the newborn, similar to the human BPD phenotype, i.e., with reduced alveolar surface area, altered septal architecture, and enlarged air spaces. In this study, we now demonstrate that treatment of prenatal inflammation with Rytvela can reverse the inflammatory cascade leading to fetal lung injury. In other words, lungs treated antenatally with Rytvela showed normal development with small and abundant alveoli, adequate for an optimal gas exchange surface.

Similarly, *in utero* exposure to LPS induced a nearly identical disrupted lung parenchyma. Rytvela significantly protected fetal lung morphogenesis from detrimental inflammation when administered up to 4h post-LPS in our model with single exposure (Figure 18), and up to 0.5h post-LPS in our model with double LPS exposure (Figure 19). These results reinforce the critical role of IL-1β in the pathogenesis of BPD and the need to rapidly address pathological antenatal inflammation to positively impact fetal pulmonary health.

BPD is a prevalent severe complication of IUGR and PTB with non-negligible long-term consequences. Currently, neonatologists have insufficient therapeutic tools to prevent and treat BPD. Here we propose a very interesting, safe, and potent therapeutic solution to improve neonatal pulmonary health by reversing pathological inflammatory phenomena *in utero*.

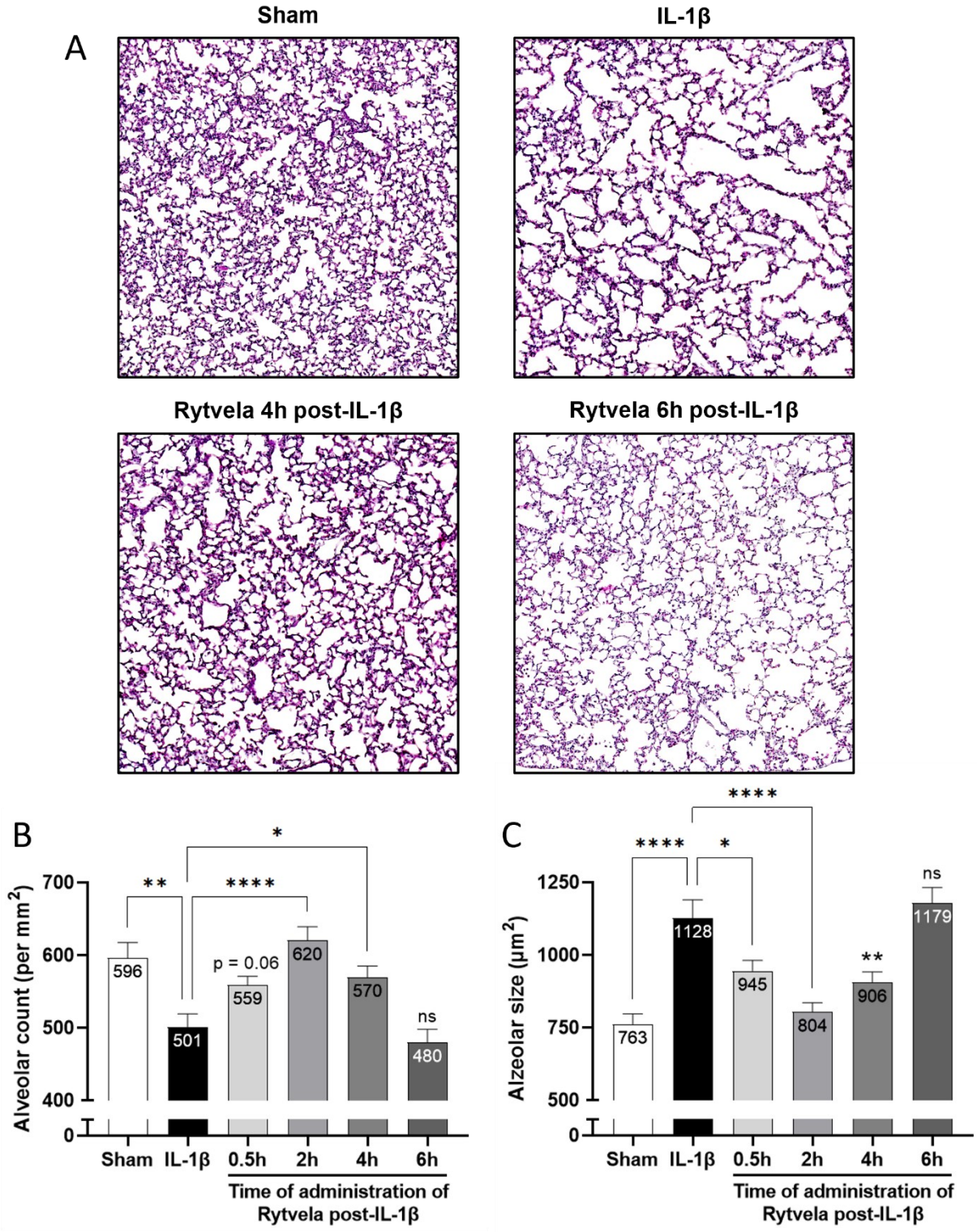

Figure 17 Lung injury induced by antenatal exposure to IL-1β is prevented with the administration of Rytvela after inflammatory onset. (**A**) Representative image of the lung parenchyma stained with H&E. Scale of images: 1000 X 1000 µm. (**B–C**) Measurement of alveolar count (B), and alveolar size (C), performed on 2-6 region of interest (ROI) per tissue section using our Python script designed by Reuben. Lungs were collected on 2 pups per litter at PT7, from 4-6 dams per group. *n* = 17-31 ROI per group. Values are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001 by one-way ANOVA with Dunnett's test.

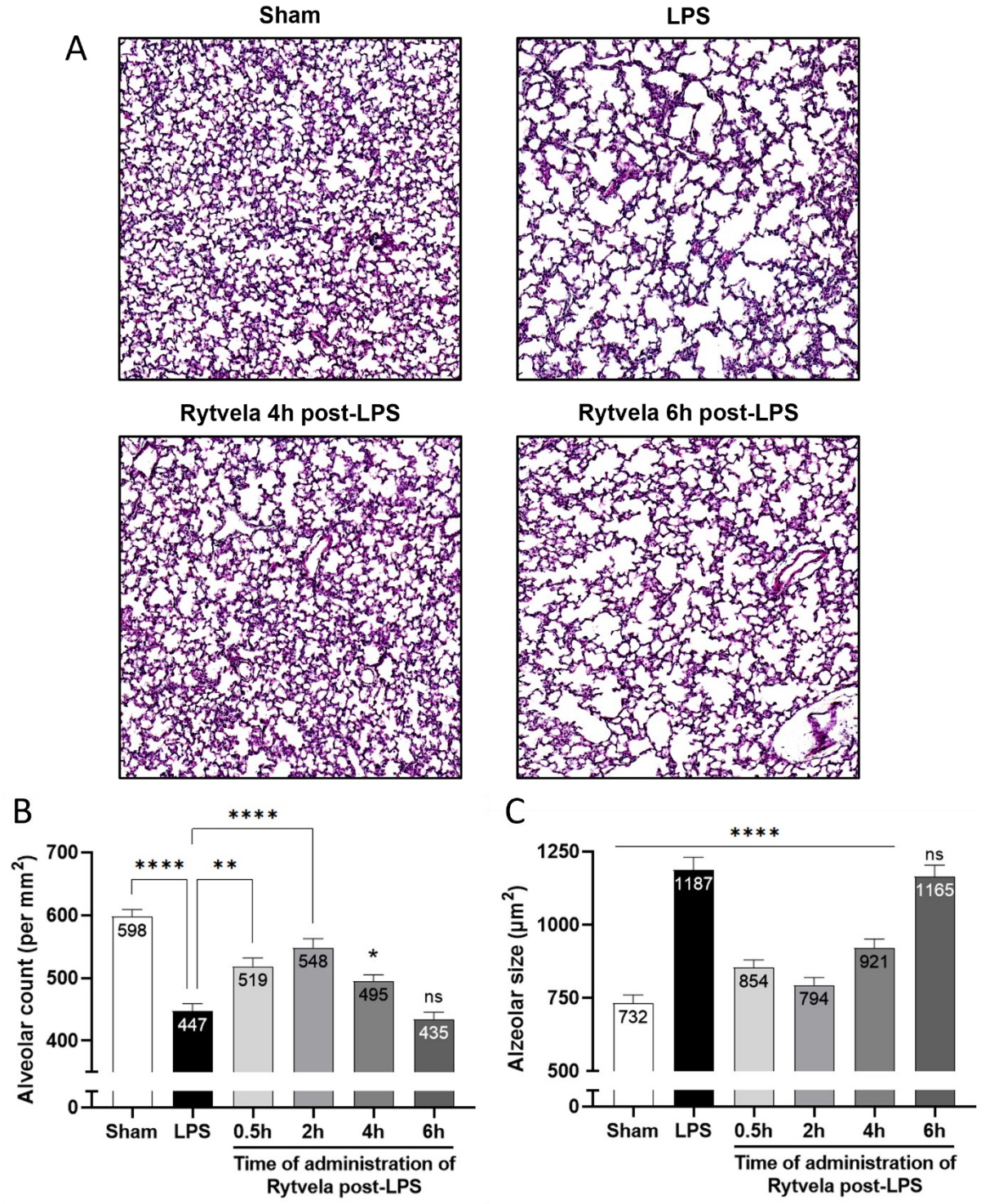

Figure 18 Lung injury induced by antenatal exposure to LPS is prevented with the administration of Rytvela after inflammatory onset. (**A**) Representative image of the lung parenchyma stained with H&E. Scale of images: 1000 X 1000 µm. (**B–C**) Measurement of alveolar count (B), and alveolar size (C), performed on 2-6 region of interest (ROI) per tissue section using our Python script designed by Reuben. Lungs were collected on 2 pups per litter at PT7, from 4-7 dams per group. *n* = 23-83 ROI per group. Values are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001 by one-way ANOVA with Dunnett's test.

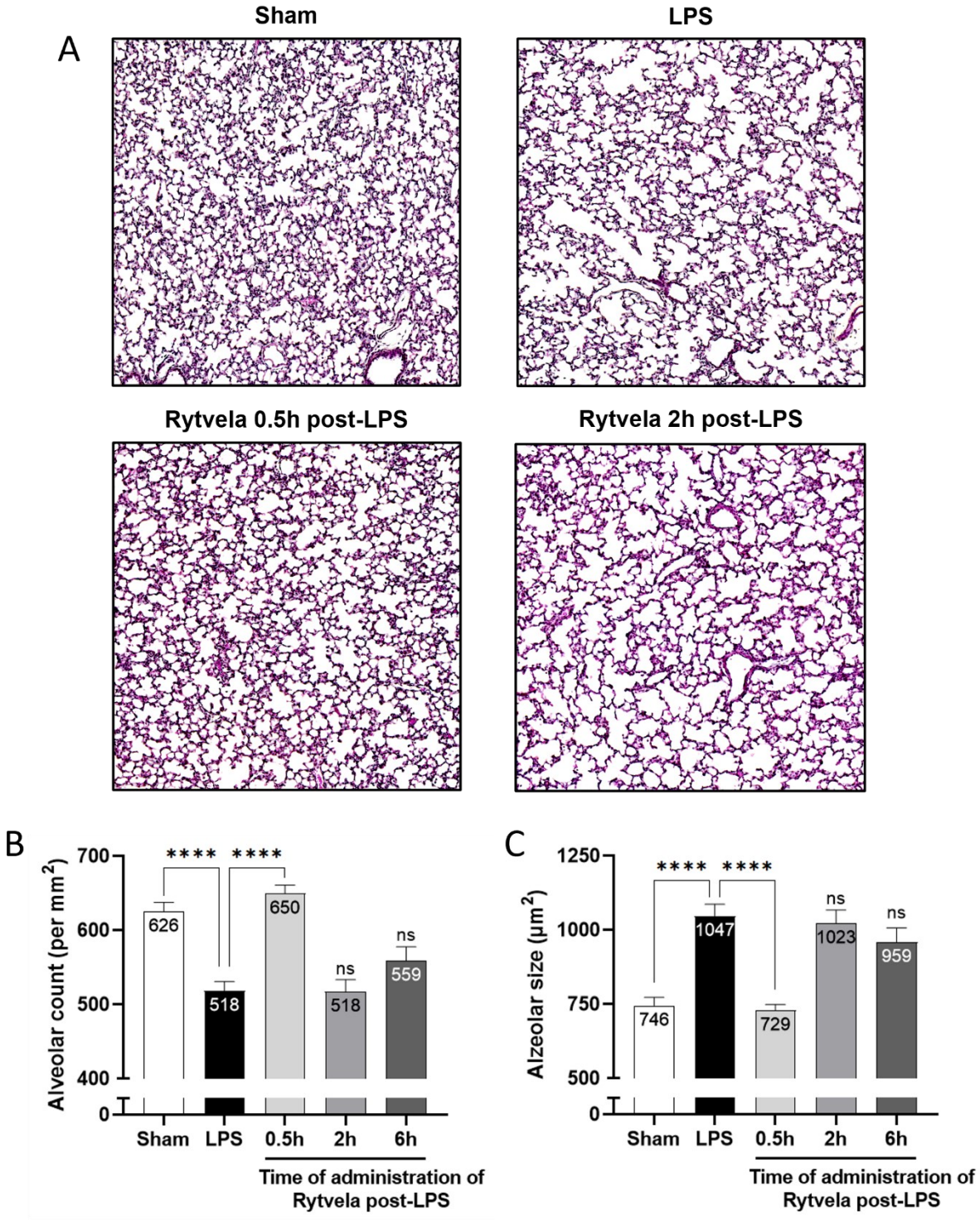

Figure 19 Lung injury induced by antenatal two-time exposure to LPS is prevented with the administration of Rytvela after inflammatory onset. (**A**) Representative image of the lung parenchyma stained with H&E. Scale of images: 1000 X 1000 µm. (**B–C**) Measurement of alveolar count (B), and alveolar size (C), performed on 2-5 region of interest (ROI) per tissue section using our Python script designed by Reuben. Lungs were collected on 2 pups per litter at PT7, from 3-7 dams per group. *n* = 16-34 ROI per group. Values are presented as mean ± SEM. ****$p < 0.0001$ by one-way ANOVA with Dunnett's test.

2.2. Rytvela protects fetal intestines in all 3 models of antenatal inflammation

The gastrointestinal tract of pups exposed to IL-1β exhibited a subnormal small diameter with shortening of the villi in the jejunum-ileum. Treatment with Rytvela up to 6h after IL-1β exposure significantly protected the pups' intestine which displayed a normal diameter with well-developed villi similar to those in our Sham group. (Figure 20).

Inflammation was shown to have a critical role in the pathophysiology of necrotizing enterocolitis (NEC), one of the principal causes of death in human premature infants. NEC is characterized by increased circulating and intestinal cytokines levels, including IL-1β, leading in its severest form to intestinal injuries such as mucosal ulceration, edema, and necrosis. NEC evolves predominantly in the terminal ileum.

In our mouse models, pups' intestinal ulceration and necrosis could not be appreciated in the inflammation groups, probably because they are very lethal consequences. Pups potentially affected probably died quickly after birth (due to the lack of a murine NICU). Deceased pups are difficult to analyze because the mother tends to eat them. In addition, it may be difficult to distinguish pre-mortem inflammatory damage from natural post-mortem tissue breakdown, especially since the intestines are fragile to proteolysis. Nevertheless, our model allowed us to appreciate a probably milder version of enterocolitis in the ~40% of pups that survived to antenatal inflammation, which resulted in atrophic intestinal phenotype. Villous atrophy is reported in inflammatory intestinal diseases like coeliac disease, autoimmune enteropathy, and other etiologically heterogeneous enteropathies. (53, 54) Thus, the phenotype obtained in our mice is consistent with intestinal damage of inflammatory origin.

In utero exposure to LPS induced a nearly identical intestinal injury in surviving pups with compromised villus integrity and smaller gastro-intestinal tract diameter. Rytvela significantly protected fetal intestine morphogenesis from detrimental inflammation when administered up to 6h post-LPS in both of our LPS models with single and double exposure (Figure 21, 22).

These results suggest that intestinal inflammatory injuries induced by in utero hostile pro-inflammatory environment, for example in the context of adverse obstetrical outcomes like IUGR and PTB, can be prevented by antagonizing IL-1 with Rytvela.

Interestingly, the intestine seemed to resist longer to antenatal inflammatory insult compared with the lungs, as a delayed treatment with Rytvela (up to 6h after the initial inflammatory insult) was effective in protecting intestinal integrity in all 3 models (compared with the effectiveness of Rytvela in protecting the lungs primarily with prompt administration 0.5h post-double-LPS-exposure). This could potentially be explained by the accrued fragility of the immature lung tissues which are in direct contact with the pro-inflammatory amniotic fluid in these circumstances. These results importantly highlight the potential beneficial effects of Rytvela on neonates' health even with tardive administration.

It should be noted that the villi are the site of digestion and absorption of nutrients via their abundant enterocytes. Compromised villi integrity can lead to a reduction in absorptive surface area (with a reduction in functional enterocytes) and a malnutrition-like phenotype, which may impact postnatal growth and development. In Figure 16C, we can see that the fetal growth retardation noted at birth persisted over time. The pups remained small and underdeveloped after 7 days of unlimited breastfeeding (see also Figure 15A).

In short, the prognosis for infants with NEC is poor, and those who survive usually face long-term complications including poor growth. Current postnatal management may include invasive surgical procedures in ~20-50% of cases. The existing therapeutic arsenal is insufficient for optimal post-natal management of the disease. Here again, we propose a safe antenatal therapeutic alternative that significantly attenuates inflammatory bowel injury and improves postnatal growth and development.

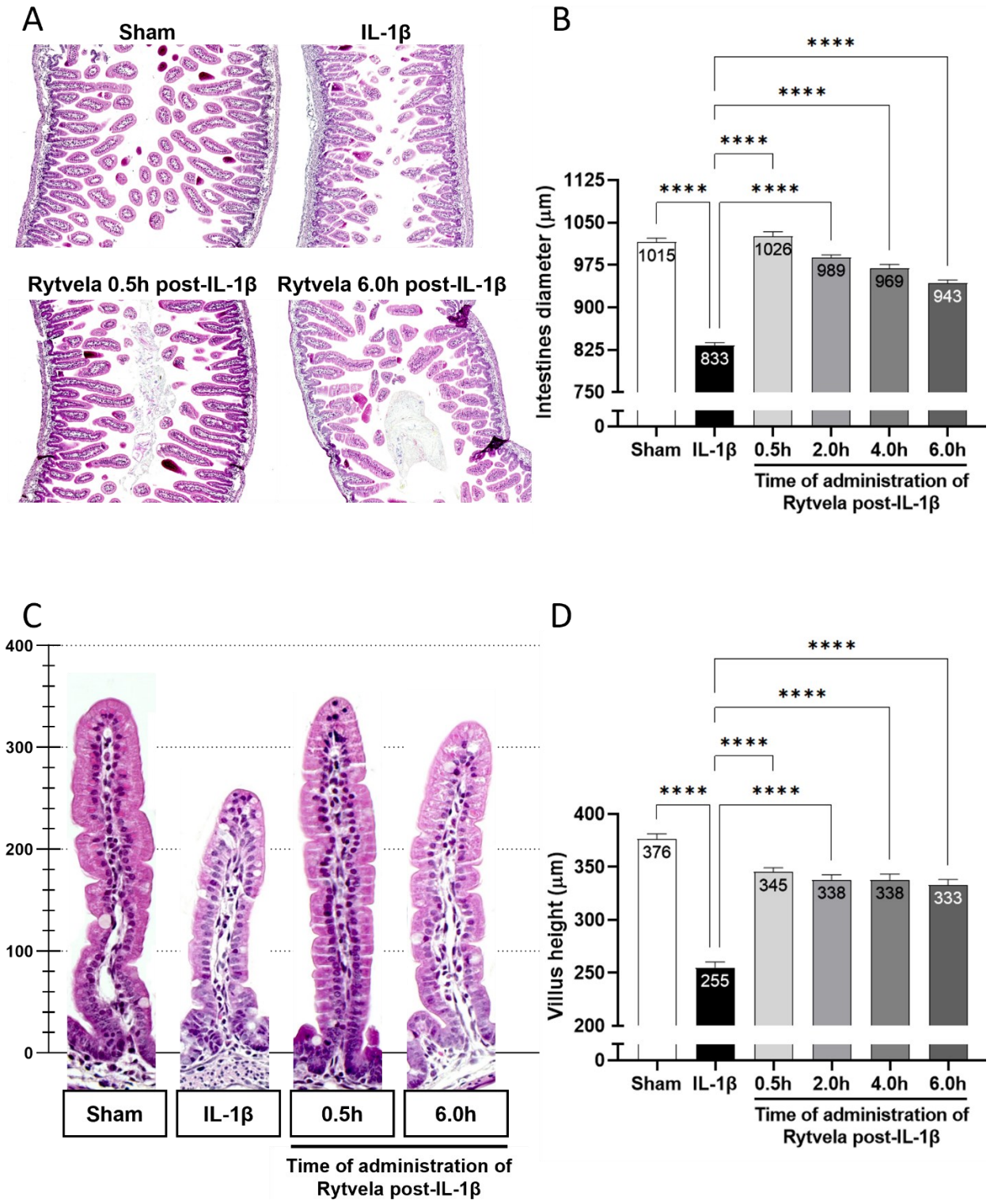

Figure 20 Intestine morphological anomalies induced by antenatal exposure to IL-1β are prevented with the administration of Rytvela after inflammatory onset. (**A**) Representative image of longitudinal sections of the pup's digestive tract at the level of the ileum, stained with H&E. Scale of images: 1000 µm height. (**B, D**) Measurement of intestinal diameter (B), and villi height (D), were performed manually using Zen3 software. (**C**) Representative image of a mean villus. Scale: 400 µm. Ileums were collected on 2 pups per litter at PT7, from 4-6 dams per group. *n* = 174-321 measures per group. Values are presented as mean ± SEM. ****$p < 0.0001$ by one-way ANOVA with Dunnett's test.

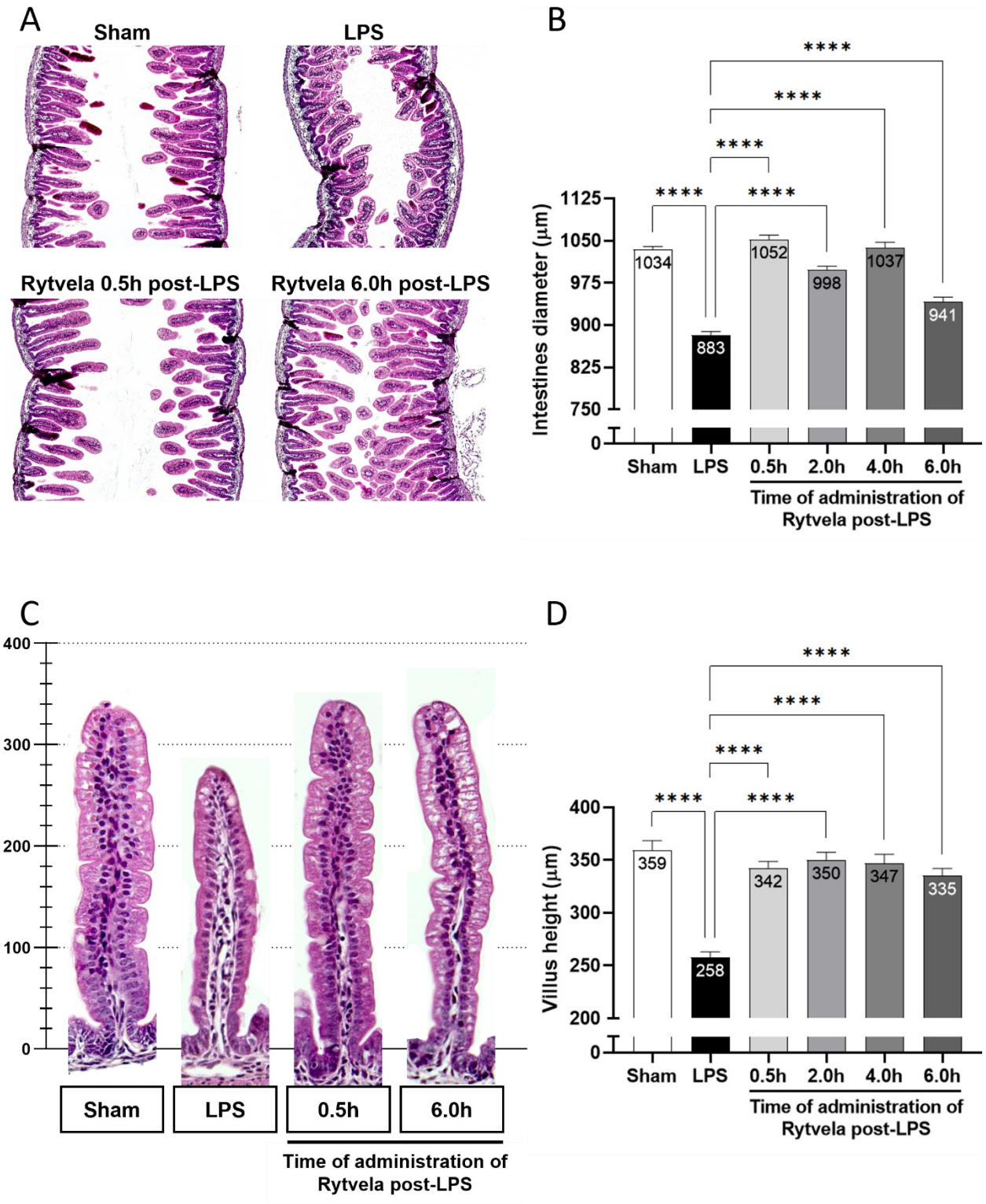

Figure 21 Intestine morphological anomalies induced by antenatal exposure to LPS are prevented with the administration of Rytvela after inflammatory onset. (**A**) Representative image of longitudinal sections of the pup's digestive tract at the level of the ileum, stained with H&E. Scale of images: 1000 µm height. (**B, D**) Measurement of intestinal diameter (B), and villi height (D), were performed manually using Zen3 software. (**C**) Representative image of a mean villus. Scale: 400 µm. Ileum were collected on 2 pups per litter at PT7, from 4-7 dams per group. *n* = 208-350 measures per group. Values are presented as mean ± SEM. ****$p < 0.0001$ by one-way ANOVA with Dunnett's test.

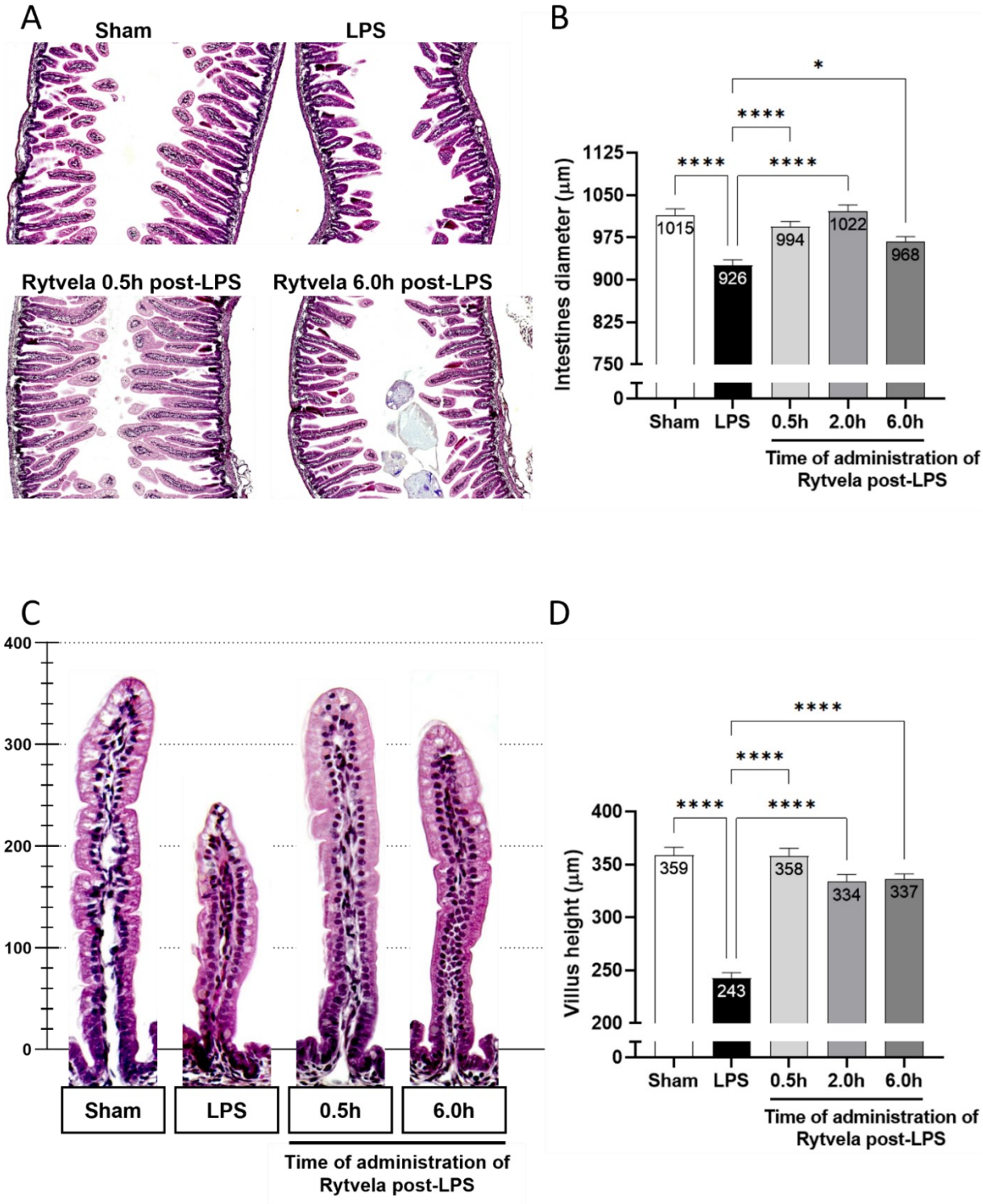

Figure 22 Intestine morphological anomalies induced by antenatal two-time exposure to LPS are prevented with the administration of Rytvela after inflammatory onset. (**A**) Representative image of longitudinal sections of the pup's digestive tract at the level of the ileum, stained with H&E. Scale of images: 1000 µm height. (**B, D**) Measurement of intestinal diameter (B), and villi height (D), were performed manually using Zen3 software. (**C**) Representative image of a mean villus. Scale: 400 µm. Ileum were collected on 2 pups per litter at PT7, from 3-7 dams per group. *n* = 88-196 measures per group. Values are presented as mean ± SEM. *$p < 0.05$, ****$p < 0.0001$ by one-way ANOVA with Dunnett's test.

2.3 Rytvela protects fetal brain in LPS model

Perinatal exposure to inflammation is also known to significantly impair brain development and cerebral angiogenesis. Analysis of brain microvasculature architecture was performed on pups from our 2-time exposure to LPS model. Pups' brains were stained with fluorescent lectin to visualize the cerebral vascular tree. In utero exposure to LPS induced significant microvascular degeneration in the cortex with a near 2-fold decrease in vascular density. Treatment with Rytvela 0.5h post-LPS efficiently protected the pups' brain as demonstrated by the preservation of vascular density (at 84% of Sham) and the associated preservation of brain mass. (Figure 23) Treatment with Rytvela 6h after the onset of inflammation modestly counteracted cerebrovascular degeneration and failed to preserve brain mass. Correspondingly, in our single exposure to LPS model, brain injury resulting in reduced brain mass was prevented by treatment with Rytvela up to 4h after the onset of inflammation (Figure 15C).

The human brain undergoes significant growth during the third trimester, with a doubling of the volume of the whole brain. This important neurogenesis is supported by enhanced angiogenesis. Conditions such as IUGR and PTB have been shown to affect brain growth, with potentially permanent functional consequences like cerebral palsy. Indeed, maternal inflammation, and consequently fetal exposure to intra-amniotic inflammation, contributes to cerebral white matter injury. Pre-exposure of the fetus to adverse intrauterine conditions has also been associated with a high incidence of intraventricular hemorrhage (IVH) suggesting increased fragility of immature blood vessels with exposition to inflammation. Deficient angiogenesis secondary to both prenatal inflammation and premature postnatal relative hyperoxic environment was also proven to be an important mechanism in neonatal encephalopathy which results in ischemia and loss of brain parenchyma.

Our mouse model recreates the context of excessive in utero inflammation, leading to PTB and secondary premature exposure to relative hyperoxic extrauterine environment. This resulted in an atypical cerebral microvascular morphology in the newborn, in line with human NE pathophysiology. The underdeveloped cerebrovascular tree was correspondingly associated with reduction in brain mass which is indicative of decay in cortical structure and function.

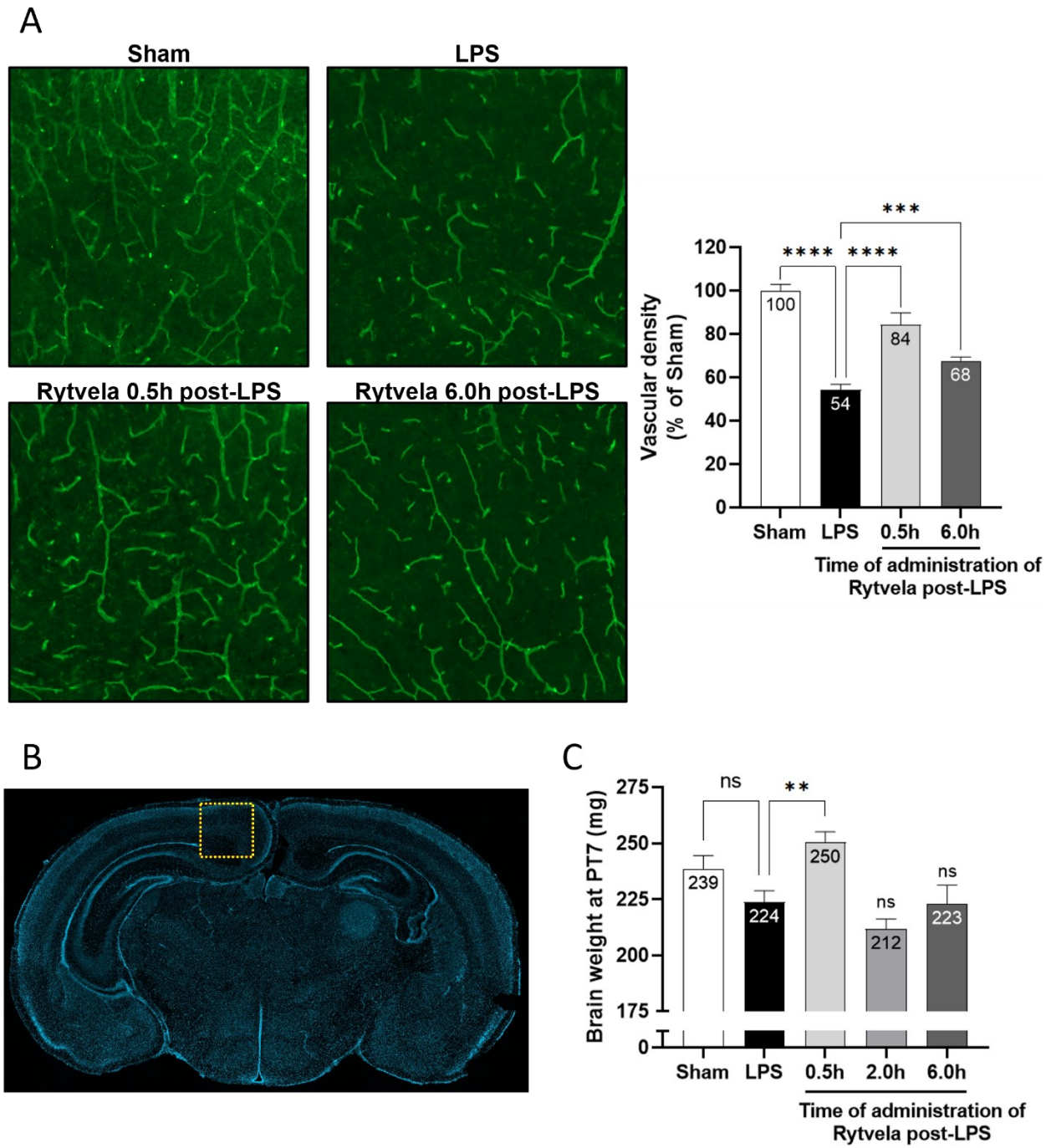

Figure 23 Microvascular degeneration in brain induced by antenatal two-time exposure to LPS is prevented with the administration of Rytvela after inflammatory onset. (**A**) Representative image of cortex vasculature and quantification of vessel density. Immunostaining for lectin (vasculature) is shown in fluorescent green. Vascular density measurements were performed on 5-25 region of interest (ROI) per group using ImageJ software. Scale of images: 750 X 750 µm. (**B**) Region quantified is represented on the full cerebral coronal section, stained with DAPI in blue. ROI of 750 X 750 µm is shown in yellow (**C**) Brain weight of pups on post-term day 7 (PT7). Brains were all collected on 2 or more pups per litter at PT7, from 3-7 dams per group. *n* = 7-18 pups per group for A-B, and 4-10 pups per group for C. Values are presented as mean ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$ by one-way ANOVA with Dunnett's test.

In this study, we now demonstrate that treatment of prenatal inflammation with Rytvela can reverse the inflammatory cascade leading to fetal brain injury. Rytvela acts in parallel by preventing PTB and thus avoiding premature toxic exposure to the relative hyperoxic extrauterine environment. This resulted in normal development of vascular tree, associated with preserved cerebral parenchymal integrity, and correspondingly preserved brain mass.

Neonatal encephalopathy (NE) is a serious complication of IUGR and PTB with irreversible lifelong consequences and for which there is still no established effective treatment. The lack of treatment is a major unmet medical need. We propose here a very interesting, safe and potent therapeutic solution to improve neonatal neurological health by reversing pathological inflammatory phenomena *in utero* and preventing preterm birth with its related postnatal brain insults.

Collectively, this data strongly supports the hypothesis that IL-1 inhibition is a promising therapeutic approach to treat lung, gut, and neural injury caused by perinatal inflammation.

In interpreting these results, it is relevant to be mindful of the differences between mice and human embryogenesis. Mice models are interesting to study for many reasons: they allow for rapid data collection, and there are extensively described in literature. Nonetheless, it remains challenging to establish a precise correlation between mouse models and humans. Mice have a shorter gestation period and are born at an earlier stage of development. Newborn mice are immature in terms of neurodevelopment, whereas humans have particularly advanced brain development at birth. (154) With regard to lung development, the alveolar stage (following saccular stage) starts *in utero* in humans (at G36) whereas in mice, it starts postnatally (at PT5). (159) Ultimately, the intent of our models is to recreate inflammation in the second trimester, which generates inflammatory damage to the immature fetal organs of the mice, generating histological phenotypes similar to what is observed in the human neonate. Nevertheless, in order to confirm the effectiveness of Rytvela in preventing inflammatory damage at different stages of embryogenesis, Rytvela has been sent to laboratories in Australia and in the United States to conduct pre-clinical experiments on larger animal models, notably sheep and monkeys, before clinical studies in humans.

CONCLUSION

In the present study, we report the ability of Rytvela, as a therapeutic agent (rather than only prophylactic), to prevent adverse obstetrical and neonatal outcomes by suppressing the inflammatory cascade primarily triggered and sustained by IL-1 signaling. IL-1 signaling is acknowledged as a common potent pathway involved in many etiological processes leading to PTB and IUGR. More precisely, we demonstrated that rapid administration of Rytvela to the mother exposed to acute/subacute, sterile/infectious inflammatory triggers prevents PTB and neonatal mortality in mice.

Concomitant with the prevention of PTB, Rytvela also reduces inflammation in the fetal compartment and in fetal organs. This is of significant importance because multiple pathologies, including IUGR, and postnatal BPD, NEC, and NE, have been strongly associated with antenatal inflammation, involving IL-1 as a key inductor. This study revealed that treatment of maternal inflammation with Rytvela had a significant positive impact on offspring development from intrauterine to extrauterine life. Rytvela preserved the growth trajectory of the pups and protected their fragile immature organs. Indeed, Rytvela prevented BPD, NEC and NE histological phenotypes in mouse neonates. Even a delayed treatment was able to provide substantial protection against antenatal inflammatory insult, notably in pups intestines and brain.

Our study corroborates the growing evidence suggesting that controlling pathologic inflammatory processes is critical to improving neonatal outcome. Maternal-fetal medicine and neonatal-perinatal medicine experts agree that there is a severe lack of therapeutic tools to prevent and treat IUGR, PTB, and associated neonatal injury. Suppression of excessive prenatal inflammation with Rytvela appears to be a safe, potent, and effective therapeutic strategy to prolong gestation and promote a healthy intrauterine environment.

Altogether, Rytvela is a promising new therapeutic prototype that contributes to a paradigm shift in the treatment of IUGR and PTB. The present study consolidates the science around Rytvela to facilitate the transfer of the technology to humans. Rytvela could contribute to saving the lives of millions of newborns and improving the health of future generations.

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