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Cervical Remodeling/ripening At Term And Preterm Delivery: The Same Mechanism Initiated By Different Mediators And Different Effector Cells

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CERVICAL REMODELING/RIPENING AT TERM AND PRETERM DELIVERY: THE SAME MECHANISM INITIATED BY DIFFERENT MEDIATORS AND DIFFERENT EFFECTOR CELLS

by

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DEDICATION

To God and my family for all their unconditional support.

To all the mothers who have experienced preterm labor.

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PREFACE

The findings presented in the dissertation have been published in the following peer reviewed manuscripts:

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Gonzalez JM, Romero R, Girardi G. Comparison of the mechanisms responsible for cervical remodeling in preterm and term labor. Journal of reproductive immunology 2013;97:112-9

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CHAPTER 1

COMPLEMENT ACTIVATION TRIGGERS METALLOPROTEINASES RELEASE INDUCING CERVICAL REMODELING AND PRETERM BIRTH IN MICE

Introduction

Successful parturition in mammalian species is characterized by two essential processes. First, the myometrium changes from a quiescent to a highly contractile state. Second, the uterine cervix undergoes a substantial remodeling process, during which a closed, rigid cervix turns into a distensible and elastic ring that stretches to allow passage of the fetus. Then, the cervix undergoes rapid repair in order to ensure protection of the reproductive tract from microbial infection. Appropriate and timely cervical remodeling is essential for successful birth. Premature cervical remodeling, which results in dilation and effacement of the cervix, can lead to preterm birth, a leading cause of perinatal mortality and morbidity. Premature birth/delivery (PTD) is one of the most significant causes of perinatal mortality and morbidity in developed countries. Preterm infants suffer morbidities including respiratory distress, intraventricular hemorrhage, and cerebral palsy, among other serious diseases.[1] These outcomes can have a life-long impact and more than $25 billion is spent each year to take care of these infants. Investigations to define the causes of PTD remain a challenge in the obstetrical field. Preterm labor is a multistage biochemical and biophysical process, during which changes in the myometrium and the cervix occur.

The uterine cervix is a complex organ that undergoes extensive changes through gestation and parturition. These changes are a common first step in preterm parturition.[2] As a gatekeeper for pregnancy, the cervix is a structural barrier that keeps the fetus inside the uterus until the end of gestation. Collagens, elastin, proteoglycans,

and hyaluronate are responsible for the full tensile strength of the cervix.[2] Ripening of the cervix is an important biological and clinical event required for a normal parturition. The process is gradual during pregnancy, with a fast final remodeling of the cervix before parturition. Increased collagen degradation and synthesis, and high activity of collagenases have been observed in the human cervix during final ripening.[3]

Improper timing of these complex changes can result in PTD, which is a major public health problem, and there has been little reduction in its incidence during the past 40 years.[4,5] This is mainly caused by our incomplete understanding of the cascade and processes that occur in the remodeling of the cervix. The characterization of the cervical changes that precede parturition in women is complicated, because it is difficult to determine antenatally which women will deliver preterm. Therefore, an animal model that best simulates the clinical scenarios can be used to investigate the mechanisms underlying cervical remodeling and PTD.[6]

Inflammation and activation of innate immunity has been associated with preterm delivery.[7] Recently, a study demonstrated that inflammatory cytokines in cervicovaginal fluid and in amniotic fluid, but not in plasma, are strongly associated with spontaneous PTD in women, suggesting that inflammation at the maternal-fetal interface, rather than systemic inflammation, may play a major role in the etiology of PTD.[8] Despite the growing association between inflammation and PTD, the mediators, receptors, and cellular participants in triggering cervical changes remain unclear. Therefore, we sought to study the mechanism or mechanisms responsible for the cervical changes underlying PTD induced by localized inflammation in mice. In this study, we induced PTD in mice by intravaginal administration of lipopolysaccharide (LPS), resembling ascending infection/inflammation, which is the most common clinical situation. We also used a

second mouse model of preterm parturition induced by progesterone antagonist RU486. Progesterone antagonists have been previously shown to cause infiltration of inflammatory cells into the cervical stroma in pregnant mice and women.[9,10]

The complement system, an important component of innate immunity, plays a pivotal role in recruiting inflammatory and immunocompetent cells contributing to various immune and inflammatory diseases. Several complications of pregnancy, including spontaneous pregnancy losses,[11,12] pre-eclampsia,[13,14] and pre-term labor[15-21] have been associated with a maternal complement activation. Importantly, complement activation initiated by the alternative pathway has been associated with preterm labor and delivery, but not labor at term.[20] Importantly, increased activation of complement components in maternal blood and the amniotic fluid has been reported in patients with preterm delivery.[15,16,19] Thus, we hypothesized that complement activation is a mediator in inflammation-induced preterm delivery in mice. The objective of the present study was to test the hypothesis that complement activation may account for the cervical changes responsible for inflammation-induced PTD in mice.

It has been reported that progesterone can reduce the rate of PTD in a high-risk population.[22-31] The biological mechanisms responsible for the protective effect of progesterone remain poorly elucidated. In addition, progesterone has been suggested to have anti-inflammatory effects,[32,33] and mice lacking progesterone receptor exhibit reproductive abnormalities characterized by inflammation.[34] Therefore, we sought to investigate if progesterone is able to decrease or prevent inflammation-induced PTD in mice. Identifying the immune effectors that mediate PTD might provide novel and important targets for the prevention and prediction of premature labor in women.

Materials and Methods

Ethics Statement

Our animal studies were performed by following the Russell and Burch “Three R's” concept of Replacement, Reduction, and Refinement to minimize animal use and pain or distress while still achieving the critical scientific objectives. All of the experiments performed were conducted in accordance with the National Institute of Health guidelines on laboratory animals and with approval from Wayne State University (protocol A 09-08-09) and York College, The City University of New York (protocol R-3-2009) committee on Animal Use and Care.

Animals

C57BL/6 timed-pregnant mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were shipped on days 10 to 12 after mating. Animals were acclimated in the animal facility for 3 to 5 days before use in these experiments. C5aR-deficient (*C5aR−/−*) females and males were also purchased from Jackson Laboratories. *C5aR−/−* females were mated with *C5aR−/−* males in our animal facility. The presence of a vaginal plug was considered as day 0 of pregnancy.

Mouse Models of Preterm Delivery

PTD Induced by LPS

In an attempt to develop a mouse model of PTD, which resembles most clinical scenarios in that localized inflammation occurs without systemic maternal illness, we administered LPS (*Escherichia coli* serotype 055:B5 (250 μg/mouse, intravaginally) on day 15 of pregnancy. This model mimics a clinical or subclinical vaginal infection/inflammation that can ascend to the cervix, the deciduas, myometrium, fetal membranes, the amniotic fluid, and thus the fetus leading to premature labor. Most of

the pregnant females (94.7%) delivered within 24 to 36 hours of LPS administration (Figure 1A). No maternal morbidity or mortality was observed in this model.

To determine whether supplementation with progesterone could prevent inflammation-induced preterm parturition, on day 15 of pregnancy the mice were randomly treated with progesterone (2 mg/mouse, s.c.; Sigma Chemicals, St Louis, MO) (*n* = 14) or an equal volume of vehicle (sunflower oil) (*n* = 5) 1 hour before LPS administration.

To elucidate the role of complement receptor C5a (C5aR), *C5aR−/−* mice (*n* = 9) received intravaginal LPS on day 15 of pregnancy.

To deplete macrophages 300 μg of anti-F4/80 (BM8; eBioscience) or isotype antibody (rat IgG2a, κ; eBioscience, San Diego, CA) was given i.p. on day 15, 4 hours before LPS injection (*n* = 4 to 6). This antibody was previously used successfully to deplete macrophages in pregnant mice.[35]

Mice from every experimental group were euthanized antepartum (12 to 18 hours after treatment) (*n* = 5 to 7 mice/group). Blood samples and cervical tissue were collected from the mice in each experimental group. Macroscopic, biomechanical, and histological evaluation of the cervix was performed. Delivery was considered preterm if it occurred within 48 hours after LPS administration (before gestational day 17).

PTD Induced by Progesterone Antagonist RU486

In this model, PTD was induced by administration of RU486 (mifepristone, Sigma Chemicals, St. Louis, MO) (150 μg/mouse subcutaneously) (*n* = 6) dissolved in dimethyl sulfoxide, as described in the literature.[36] It was previously described that RU486 increases the recruitment of immune cells to the cervix.[9,10] Mice treated with RU486 give birth within 24 hours (Figure 1A). Mice were euthanized during the antepartum (12

to 18 hours after treatment) to analyze the cervical changes that precede PTD. Gestational age-matched controls were euthanized at the same time as treated mice. Delivery was considered preterm if it occurred within 48 hours after RU486 administration (before gestational day 17).

To study the role of C5aR, *C5aR−/−* mice (*n* = 7) were treated with RU486 on day 15 as previously described. Mice from every experimental group were euthanized antepartum (12 to 18 hours after treatment). Gestational age-matched controls were euthanized at the same time as treated mice. Blood samples and cervical tissue were collected from mice in each experimental group. Macroscopic, biomechanical, and histological evaluation of the cervix was performed.

Serum C3adesArg and C5adesArg Levels

To determine whether complement activation occurs in LPS and RU486-treated mice that deliver preterm, generation of complement split products C3a and C5a were measured by the more stable metabolites C3adesArg and C5adesArg. The C3adesArg in serum was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using rat anti-mouse C3a and biotin rat anti-mouse C3a. Both antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA). A Standard curve was performed using purified mouse C3a protein (native) (BD Biosciences Pharmingen). C5adesArg was also measured by sandwich ELISA using rat anti-mouse C5a and biotin rat anti-mouse C5a. Both antibodies and mouse purified C5a protein for the standard curve were purchased from BD Biosciences.

To determine whether LPS treatment diminished progesterone levels in mice, antepartum serum progesterone was measured by Enzyme Immune assay (Cayman Chemicals, Ann Arbor, MI).

Tissue Biomechanics

The stiffness of the cervix was evaluated by measuring the elastic modulus. The cervix were excised longitudinally and mounted to tensile testing equipment with a calibrated mechanical drive attached to a force transducer (Com-Ten, Pinellas Park, FL). The length of the cervix was increased isometrically in 10-mm increments per minute. The amount of force required to distend the cervix and the tension exerted by the stretched tissue were recorded. The length was increased until either forces exerted by the tissue reached a plateau or the tissue tore. Force was plotted as a function of cervical length. The slope of the linear portion of the force-strain curve was computed as an index of tissue stiffness and elasticity (elastic modulus). A steeper slope indicates increased resistance to stretch, and therefore decreased elasticity (elastic modulus). The data were recorded using data acquisition Com-Ten software.

Immunohistochemistry and Immunocytochemistry

For immunohistochemical studies, cervical tissues were frozen in OCT compound, and cut into 10-μm sections. These sections were stained for C3 with rabbit anti-mouse C3 (2 μg/mL; LifeSpan Biosciences, Seattle, WA), neutrophils were stained with rat anti-mouse granulocyte RB6-8C5 mAb (5 μg/mL; BD Biosciences Pharmingen), and macrophages were stained with F4/80 (10 μg/mL; Novus Biologicals Inc, Littleton, CO). Matrix metalloproteinase (MMP)-9 was detected in frozen cervical sections using a rabbit polyclonal anti-mouse MMP-9 antibody (10 μg/mL; Abcam, Cambridge, MA). MMP-2 was detected in using a rabbit polyclonal anti-mouse MMP-2 antibody (3 μg/mL; Abcam, Cambridge, MA). Collagen I distribution was determined using a polyclonal antibody against collagen type I (10 μg/mL; ACRIS Antibodies, GmbH, Herford, Germany). Horseradish peroxidase-labeled specific secondary antibodies and 3,3′-

diaminobenzidine as substrate were used to develop the respective reactions.

Masson's Trichrome staining was also performed to detect collagen fibers distribution in cervical frozen sections. The collagen fibers stained blue and the background stained red. Four slides per animal were stained and 6 to 8 animals were studied in each experimental group.

In situ Zymography

MMP-9 activity and MMP-2 activity were measured by i*n situ* zymography as previously described.[37] Briefly, 10-µm-cervix sections were washed in PBS and then incubated for 2 hours in 20 µg/mL DQ-labeled gelatin, DQ-collagen I, or DQ collagen IV (Invitrogen, Carlsbad, CA) in 50 mmol/L Tris-HCl, 50 mmol/L NaCl, 10 mmol/L $CaCl_2$, pH 6.8 at 37°C in the dark. In parallel, control sections were pre-incubated with buffer containing 10 mmol/L EDTA followed by 2-hour incubation in DQ-gelatin or DQ-collagen solution supplemented with 10 mmol/L EDTA to indicate the contribution of metalloproteinases. The reaction was stopped by removing the substrate solution followed by 10 minutes incubation in 4% paraformaldehyde PBS. Finally, mounting medium supplemented with DAPI (Vector Laboratories, Burlingame, CA) was applied. Sections were observed under a fluorescence microscope (Nikon Eclipse 50i; Nikon Inc., Melville, NY) and photographs were taken using a Nikon DigiSight Color Digital Camera System and NIS-Elements Research Imaging Software. Increased fluorescence indicates increased gelatin and collagen degradation by MMPs. Each experimental group consisted of 6 to 9 mice and four tissue sections per animal were studied.

Preparation of Cervical Tissue Lysates for Gelatin Zymography

Cervical tissues were dissected immediately after euthanasia, and 1 g of tissue

was homogenized in 1 mL of lysis buffer consisting of 0.1 M Tris-HCl, pH 6.8, 1 M urea, 1% nonyl phenoxypolyethoxylethanol (NP)-40, 10 mmol/L EDTA, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), and proteinase-inhibitor mix, using a homogenizer. All lysates were centrifuged at 15,000 g for 30 minutes, and colorimetric protein detection was performed in the supernatants using the BCATM Protein assay kit (Pierce, Rockford, IL). Samples of equal protein content (25 µg) were mixed with fivefold concentrated Laemmli buffer separated by electrophoresis on 10% SDS-polyacrylamide gels containing 0.8 mg/mL gelatin in a nonreducing condition. After electrophoresis, the gel was rinsed twice for 10 minutes in 2.5% Triton-X-100 to wash off the SDS and rinsed twice for 10 minutes in water to remove Triton-X-100. The gel was then incubated with an incubation buffer containing 1% Tween 20, 10 mmol $CaCl_2$, 1 µmol $ZnCl_2$, and 50 mmol Tris-Cl (pH 7.5) at 37°C for 18 hours, and stained with Bio-Safe Coomassie Stain solution (Bio-Rad, Hercules, CA). Purified Pro-MMP-9 (R&D Systems, Minneapolis, MN) was used for identification. Three independent experiments with different mice were performed.

Production of MMP-9 Proenzyme in vitro

Splenocytes from C57BL/6 female mice were incubated for 12 and 24 hours in culture medium supplemented with 10% inactivated fetal bovine serum. Nonadherent cells were removed and adherent cells (>95% peroxidase-positive) were incubated in culture medium supplemented with 10% inactivated fetal bovine serum, with the addition of the following stimuli: none (control); C5a (10 nmol/L); C5a (10 nmol/L) plus progesterone (10 nmol/L). After 12 and 24 hours, culture supernatants were collected and analyzed for MMP-9 proenzyme by ELISA (R&D Systems).

A group of macrophages was incubated on slides with increasing concentrations

of progesterone (10, 20, and 40 nmol/L). After 12 hours incubation, macrophages were tested for C5aR synthesis by RT-PCR and expression by IHC. For IHC, cells were fixed in 5% paraformaldehyde and stained with anti-C5aR antibody (20 μg/mL Hycult Biotech; Cell Sciences Inc., Canton, MA) followed by secondary antibody labeled with horseradish peroxidase. Some macrophages were permeabilized before staining with Tween 20 (0.5%). The histochemical reaction was developed with DAB. The DAB staining was quantified using the NIS-Elements Research Imaging software (Nikon Inc. Tech Co., Ltd., Tokyo, Japan). Exposure time was fixed and the camera settings were kept constant. The macrophages were segmented based on red/green/blue intensity, and the area was restricted to eliminate debris. Measurements data per object were calculated using the following parameters: sum density (the sum of individual optical densities of each pixel in the area being measured) and mean density (statistical mean of density values of pixels). NIS-Elements BR (Nikon Inc., Tokyo, Japan) uses density calibration curves for evaluation of these parameters. There were 25 macrophages per slide that were analyzed in each experimental condition.

Quantitative RT-PCR

To determine whether C5a differentially regulated the expression of MMP-9 in macrophages, we studied the synthesis of MMP-9. RNA was harvested from macrophages with RNeasy Mini Kit (Qiagen, Valencia, CA) and 1 μg of total RNA was reverse transcribed. Relative quantification of gene expression was performed by real-time PCR using The Biomark System (Fluidigm San Francisco, CA). Primer sequences mouse glyceraldehydes-3-phosphate dehydrogenase and mouse MMP-9 were obtained from Applied Biosystems (Foster City, CA). Relative expression was normalized for levels of GAPDH. The generation of only the correct size amplification products was

confirmed using agarose gel electrophoresis.

To investigate the effects of progesterone on C5aR expression in macrophages, we also performed qRT-PCR as previously described. The primers for C5aR were also obtained from Applied Biosystems (Foster City, CA).

Statistical Analysis

Data are expressed as mean ± SD. After confirming that the data were normally distributed (Kolomogorov-Smirnov test of normalcy), statistical analyses were conducted using Student's *t*-test to compare differences in means. Associations were considered to be statistically significant if the value of $P < 0.05$. Data were processed using SigmaStat (version 3.1), statistical program for Windows.

Results

Collagen Degradation in Cervical Remodeling Induced by LPS and RU486

Mice treated with LPS or RU486 delivered preterm. Parturition in these mice occurred before gestational day 17 (Figure 1A). To elucidate the mechanism responsible for PTD, we investigated if remodeling of the cervix occurs in these mice.

Collagen I and III are the predominant component of the cervix extracellular matrix and gives tensile strength to it.[2,38] Collagenases of the MMP family degrade collagen softening of the cervix (in a process called cervical remodeling) and prepare it for dilation and delivery.[3] Macroscopic examination of the cervix in LPS- and RU486–treated mice (Figure 1B) antepartum showed increased distensibility and elasticity when compared to firm and rigid cervix observed in gestational age match controls (Figure 1B, left panel). Biomechanical studies of the cervix confirmed these results. The slope of the ascendant part of the regression line of the stress-strain curve (elastic modulus) was decreased in the cervical tissue of LPS- and RU486-treated mice when compared

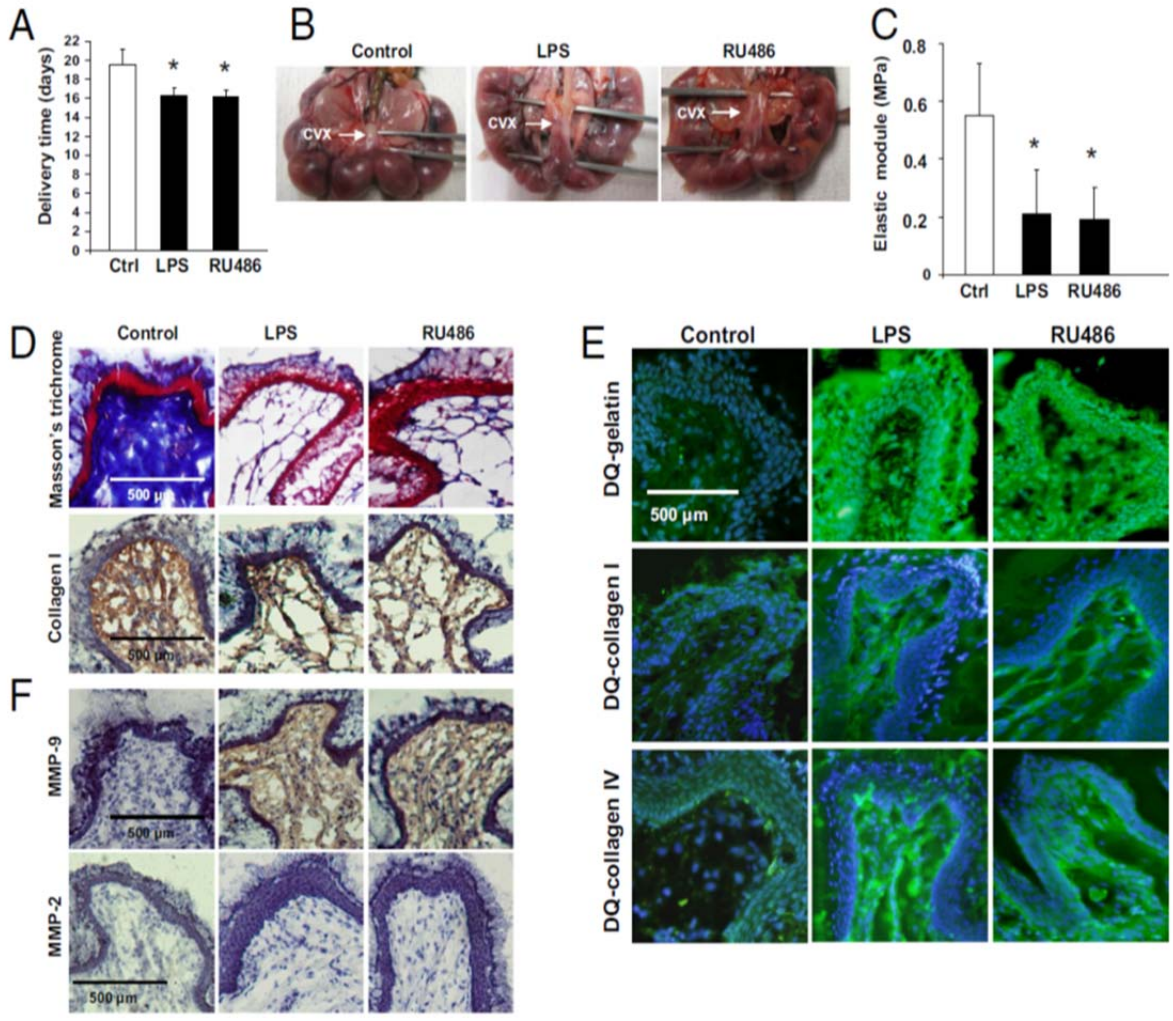

Figure 1. Cervical remodeling and preterm labor/delivery (PTD) in LPS- and RU486-mice. A: Delivery time in control and LPS- or RU486-treated mice (*n* = 5 to 7 mice/group). Significantly different from control, **P* < 0.05. **B:** Gross evaluation of the cervix. **Arrows** identify the posterior side of the cervix. Note the increased distensibility observed in LPS- (**middle panel**) and RU486-treated mice (**right panel**) when compared to the firm and rigid cervix of control mice (**left panel**). **C:** Biomechanical studies of the cervix. The elastic modulus was decreased in the cervical tissue of LPS- and RU486-treated mice when compared to control mice, suggesting a decrease in the stiffness of the cervix in the mice that deliver preterm. **D:** Collagen staining. The collagen was determined by Masson's trichrome staining (**upper panels**) and by IHC using a specific antibody that reacts with mouse collagen type I (**lower panels**). Dense network of collagen fibers was observed in control cervix (**left panels**). Disperse thin fibers of collagen were observed in the cervix of LPS- (**middle panels**) and RU486-treated mice (**right panels**). **E:***In situ* zymography using DQ gelatin (**upper panel**), DQ collagen type I (**middle panel**), and DQ collagen type IV (**lower panel**). Increased fluorescence (increased gelatin and collagen I and IV degradation) is present in the cervix of LPS- (**middle panel**) and RU486-treated mice (**right panels**). Weak fluorescence (low gelatin and collagen degradation) is observed in control cervical tissue (**left panels**). **F:** Detection of matrix metalloproteinase (MMP)-2 and MMP-9 in cervical tissues by IHC. Increased MMP-9 staining was observed in the cervix of LPS- (**middle upper panel**) and RU486-treated mice (**right upper panel**) when compared to control mice (**left upper panel**). On the other hand no increase in staining for MMP-2 was observed in the cervix of LPS- (**middle lower panel**) and RU486-treated mice (**right lower panel**). The weak staining for MMP-2 in these mice was comparable to control mice (**left lower panel**).

to control mice, suggesting a decrease in the stiffness of the cervix in mice that deliver preterm (Figure 1C). Masson's trichrome (TC) staining revealed a decreased number of collagen fibers in the cervix of mice treated with LPS or RU486 antepartum (Figure 1D, middle and right upper panels) when compared to the high density of collagen fibers observed in control mice (Figure 1D, left upper panel). Immunohistochemical studies with specific antibodies against mouse collagen I showed a similar distribution pattern to the TC staining (Figure 1D, lower panels). Collagen packing was disorganized, collagen fibers looked thinner, and greater spacing between fibers were observed in LPS- and RU486-treated mice (Figure 1D, middle and right lower panels) when compared to age-matched controls (Figure 1D, left lower panel).

Decreased density in collagen fibers was associated with increased cervical MMP activity (Figure 1E). *In situ* zymography using DQ gelatin and DQ collagen I and IV as substrate revealed an increase in activity of MMPs with gelatinolytic and collagenolytic activity in the cervix of mice treated with LPS or RU486 (Figure 1E). Collagen is degraded by the sequential action of many MMPs to gelatin, and subsequently gelatin is degraded by MMPs. Both MMP-9 and MMP-2 have the capacity to cleave collagen I, collagen IV, and gelatin.[39] Robust MMP activity (green fluorescence) was observed in the cervix of LPS- and RU486-treated mice incubated with DQ gelatin and DQ collagen I and IV (Figure 1E) in contrast with the weak fluorescence (diminished MMP activity) observed in the cervix of control mice (Figure 1E, left panels). Taken together, these data suggest that MMP-9 and/or MMP-2 might be responsible for the degradation of the cervical matrix during the antepartum period, decreasing cervical tensile strength, and leading to PTD in LPS- and RU486-treated mice.

Immunohistochemical studies identified MMP-9 as the main collagenase involved in cervical remodeling in LPS- and RU486-treated mice that deliver preterm. Increased MMP-9 staining (Figure 1F, middle and right upper panels), but not MMP-2 (Figure 1F, middle and right lower panels) was observed in the stroma and epithelium of mice with inflammation-induced preterm birth compared to age-matched controls (Figure 1F, upper and lower right panels).

Complement Activation in LPS- and RU486-Treated Mice

Knowing that complement activation plays a crucial role in many pregnancy complications, we investigated if complement activation is a mediator in the pathogenesis of the cervical changes that lead to PTD in LPS- and RU486-treated mice. In accordance with our hypothesis, increased C3 deposition was observed in the cervical columnar epithelium in LPS- and RU486-treated mice (Figure 2A) when compared to gestational age-matched controls (Figure 2A). In addition, increased C3adesArg levels were measured in serum of LPS- and RU486-treated mice during antepartum compared to control mice (Figure 2B). Complement split product C5adesArg was also increased in LPS- and RU486-treated mice when compared to controls (Figure 2C).

Inflammatory Cells in the Cervical Tissue

It has been suggested that inflammatory cells play an important role in the cervical changes before delivery at term. Thus, we investigated if inflammatory cells are present during cervical remodeling in preterm delivery induced by LPS and RU486. Immunohistochemical studies with anti-F480 antibodies showed increased macrophages infiltration in the cervical stroma, epithelium, and mucus in LPS- and RU486-treated mice compared to gestational age-matched controls (Figure 2D). The

presence of macrophages in the cervix of mice that deliver preterm suggests that macrophages may be involved in the cervical changes that precede PTD.

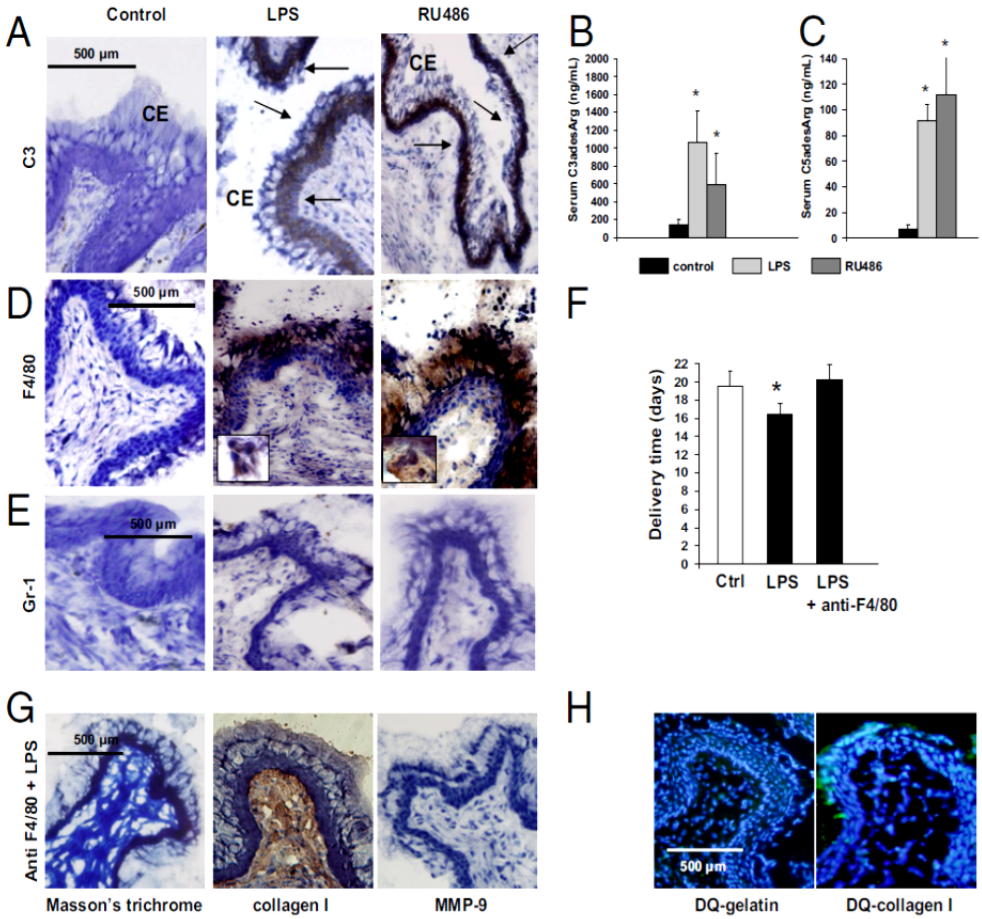

Figure 2. Complement activation and macrophages/monocytes infiltration in the cervix of LPS- and RU486-treated mice. A: Immunohistochemical determination of C3 in cervical tissue. Arrows indicate C3 deposition (brown staining) in the columnar epithelium (CE) in LPS- (**middle panel**) and RU486-treated mice **(right panel**). No complement deposition was observed in the cervical tissue of the control mice (**left panel**). Original magnification is ×20. **B**: Serum C3adesArglevels in control, LPS-, and RU486-treated mice. Statistically different from control, *P < 0.05 (n = 5 to 6 mice/group). **C**: Serum C5adesArglevels in control, LPS-, and RU486-treated mice. Statistically different from control, *P < 0.05 (n = 5–6 mice/group). **D**: F4/80 staining in cervical tissue. Increased macrophage infiltration (brown color) was observed in the cervical stroma, epithelium, and mucus of LPS- (**middle panel**) and RU486-treated mice (**right panel**). Few macrophages were observed in the cervix of age-matched control mice (**left panel**). Original magnification is ×20. The inserts in the **middle** and **right panels** include macrophages shown at a higher magnification. **E**: Gr-1 staining in cervical tissue. Few Gr-1+ neutrophils cells were found in the cervical stroma of control (**left panel**), LPS- (**middle panel**), and RU486-treated mice (**right panel**). Original magnification is ×20. **F**: Delivery time in control, LPS-, and LPS plus anti-F4/80 (n = 4–6 mice/group). Significantly different from control, *P < 0.05. **G**: Trichrome (TC) staining for collagen I and for matrix metalloproteinase (MMP)-9 in cervical tissue of LPS-treated mice depleted of macrophages with anti-F4/80. Abundant collagen fibers were shown with TC staining (left panel) and by IHC with antibodies against collagen I (**middle panel**). Staining for MMP-9 was negative in F480 plus LPS-treated mice (**right panel**). ***H****:In situ* zymography using DQ-gelatin and DQ-collagen I as substrate in LPS-treated mice pretreated with anti-F4/80. Gelatin and collagen I degradation was not observed in cervical tissue from F4/80 plus LPS-treated mice. Original magnification is ×20**.**

Few positive Gr-1 cells were observed in the cervix of control, LPS- and RU486-treated mice (Figure 2E) suggesting that neutrophils might not be involved in the cervical remodeling that precedes PTD in LPS- and RU486-treated mice.

Macrophages Depletion Prevents PTD in LPS-Treated Mice

To examine the relative importance of macrophages in LPS-induced cervical changes and preterm delivery, we depleted macrophages by treating mice with anti-F4/80 antibodies. In the absence of macrophages, treatment with LPS did not cause preterm birth (Figure 2F) nor were there inflammatory infiltrates in the cervix (data not shown). Furthermore, without macrophages infiltration there was less collagen degradation, as shown in Figure 2G in which TC staining and IHC with anti-collagen I antibodies showed a dense network of collagen fibers comparable to control mice (Figure 1D). Decreased MMP-9 activity (Figure 2G) and decreased proteolytic activity against gelatin and collagen I (Figure 2H) was also observed in LPS-treated mice depleted of macrophages. Diminished collagen degradation and MMP-9 activity suggest that macrophages may play a role in cervical remodeling that leads to PTD in LPS-treated mice. LPS-treated mice that received isotype control antibody delivered within 24 to 36 hours (data not shown).

C5a and C5aR Interaction Is a Critical Mediator of LPS- and RU486-Induced Preterm Delivery

Because receptors for anaphylotoxin C5a (C5aR) are widely expressed in inflammatory cells and endothelial cells among others,[40,41] we hypothesized that C5a and C5aR interactions mediate cervical remodeling and PTD in LPS- and RU486-treated mice. To test this hypothesis we performed studies in *C5aR−/−* mice. Untreated

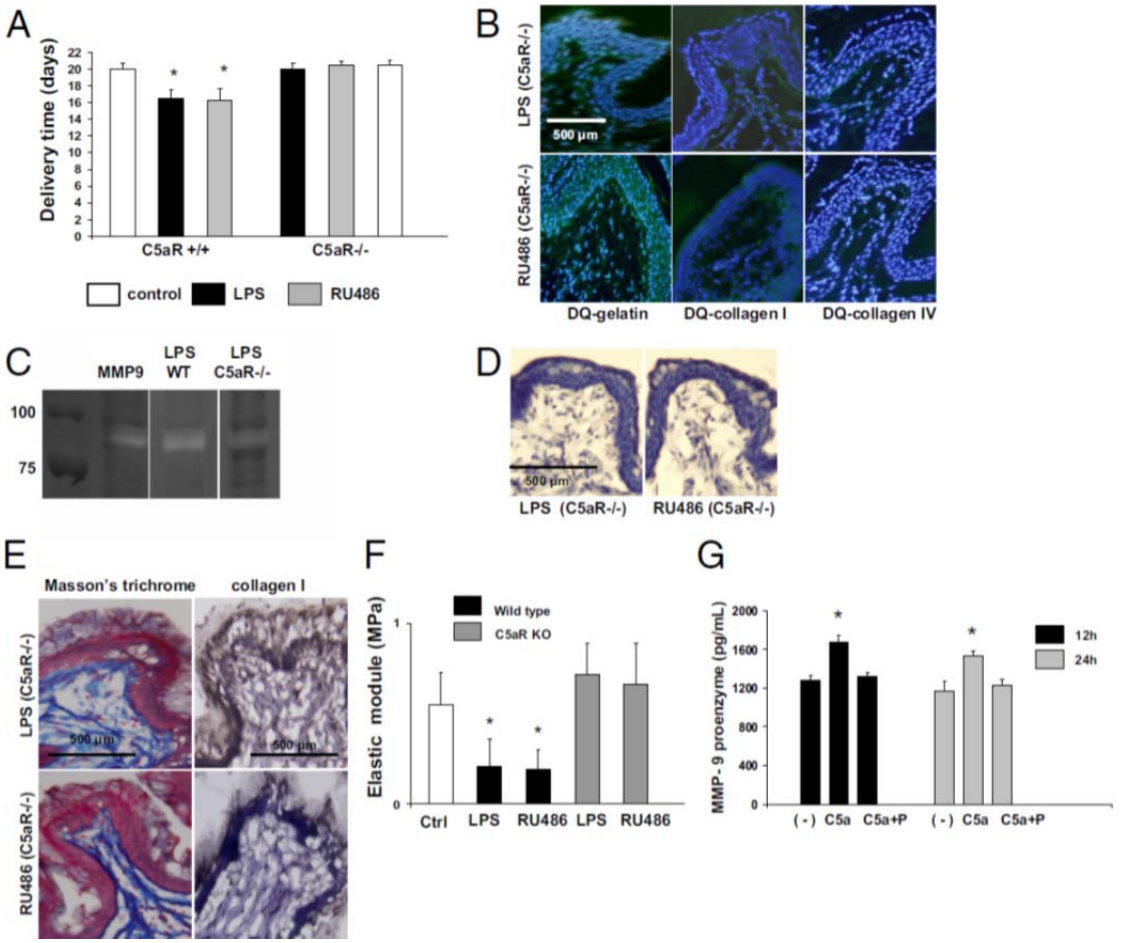

Figure 3. C5aR deficiency and progesterone prevents preterm labor/delivery (PTD). A: Delivery time in control and LPS- or RU486-treated, wild-type, *C5aR+/+* and *C5aR−/−* mice (*n* = 5 to 7 mice/group). Significantly different from control, **P* < 0.05. **B:***In situ* zymography using DQ gelatin (**left panels**), DQ collagen I (**middle panels**), and DQ collagen IV (**right panels**). In contrast to the increased fluorescence (increased gelatin and collagen degradation) observed in the cervix of LPS- (Figure 1E, middle panels) and RU486-, wild-type treated mice (Figure 1E, right panels), gelatin and collagen degradation was not observed in LPS- (**upper panels**) and RU486-treated, C5aR−/− mice (**lower panels**) comparable to control cervical tissue (Figure 1E, left panels). **C:** Gel zymography of nonreduced cervical tissue lysates. Purified matrix metalloproteinase (MMP)-9 was used as a control. Increased MMP-9 was observed in wild-type (WT) mice treated with LPS. **D:** Immunohistochemical detection of MMP-9 revealed weak staining for MMP-9 in LPS- (**left panel**) and RU486-treated, C5aR−/− mice (**right panel**). **E:** Collagen detection by trichrome staining and IHC. Dense network of collagen fibers was observed in the cervix of LPS- (**upper panels**) and RU486-treated (**lower panels**) *C5aR−/−* mice in contrast with the disperse thin fibers of collagen observed in the cervix of LPS- and RU486-treated, wild-type, *C5aR+/+* mice (Figure 1D, middle and right panels, respectively). **F:** Biomechanical studies of the cervix. The elastic modulus (measure of the tissue stiffness) was decreased in the cervical tissue of LPS- and RU486-, wild-type treated mice when compared to control mice. Decreased elastic module was not observed in LPS- and RU486-treated C5aR−/− mice, indicating an increase in the stiffness of the cervix in these mice. Elastic module values in LPS- and RU486-treated mice were comparable to age-matched control mice (*n* = 4 mice/group). Significantly different from control mice (Ctrl), **P* < 0.05. **G:** Complement activation triggers release of pro-MMP-9 by murine macrophages *in vitro*. Splenic macrophages were stimulated with C5a and C5a plus progesterone (P). Culture supernatants were collected after 12 and 24 hours and analyzed for MMP-9 by enzyme-linked immunosorbent assay (*n* = 4 experiments/group; **P* < 0.05 versus untreated cells).

C5aR−/− (control) mice have normal pregnancies and give birth at day 20 to day 21, similar to C5aR+/+, wild-type mice (Figure 3A). In wild-type, *C5aR*$^{+/+}$ mice, LPS, and RU48-induced PTD (Figure 3A). Neither LPS nor RU486 treatment induced PTD in *C5aR*$^{-/-}$ mice (Figure 3A). Increased MMP activity observed in LPS- and RU486-treated, wild-type, *C5aR+/+* mice (Figure 1E) was not observed in *C5aR−/−* mice (Figure 3B). Gelatin zymography confirmed these results (Figure 3C). A diminution in MMP-9 activity was observed in the cervix of LPS-treated, *C5aR−/−* mice (Figure 3C, lane 4) when compared to wild-type, *C5aR+/+* mice (Figure 3C, lane 3). Staining for MMP-9 was negative in cervical tissue from LPS- (Figure 3D, left panel) and RU486-treated mice (Figure 3D, right panel). As expected, the absence of MMP9 expression and activity in LPS- or RU486-treated, *C5aR−/−* mice (Figure 3, B and D) was correlated with diminished collagen degradation. Abundant collagen fibers were observed in the cervix of LPS- (Figure 3E, upper panels) and RU486-treated, *C5aR−/−* mice (Figure 3E, lower panels). The histological studies correlated with the biomechanical studies of the cervix. Decreased distensibility (increased elastic module) values were observed in the cervix of *C5aR−/−* mice treated with LPS or RU486 when compared to wild-type mice (Figure 3F). These data suggest that C5aR is required for preterm cervical remodeling and PTD in LPS- and RU486-treated in mice.

Progesterone Prevents Cervical Remodeling and PTD in LPS-Treated Mice

As progesterone withdrawal has been related with PTD, we measured progesterone levels in LPS-treated mice. We found that serum progesterone levels in LPS-treated mice antepartum were not different from age-matched control mice (42 ± 7 ng/mL in LPS-treated mice (n = 6) versus 39 ± 6 ng/mL in control mice (n = 7). Because some studies reported that progesterone and 17 OH hydroprogesterone

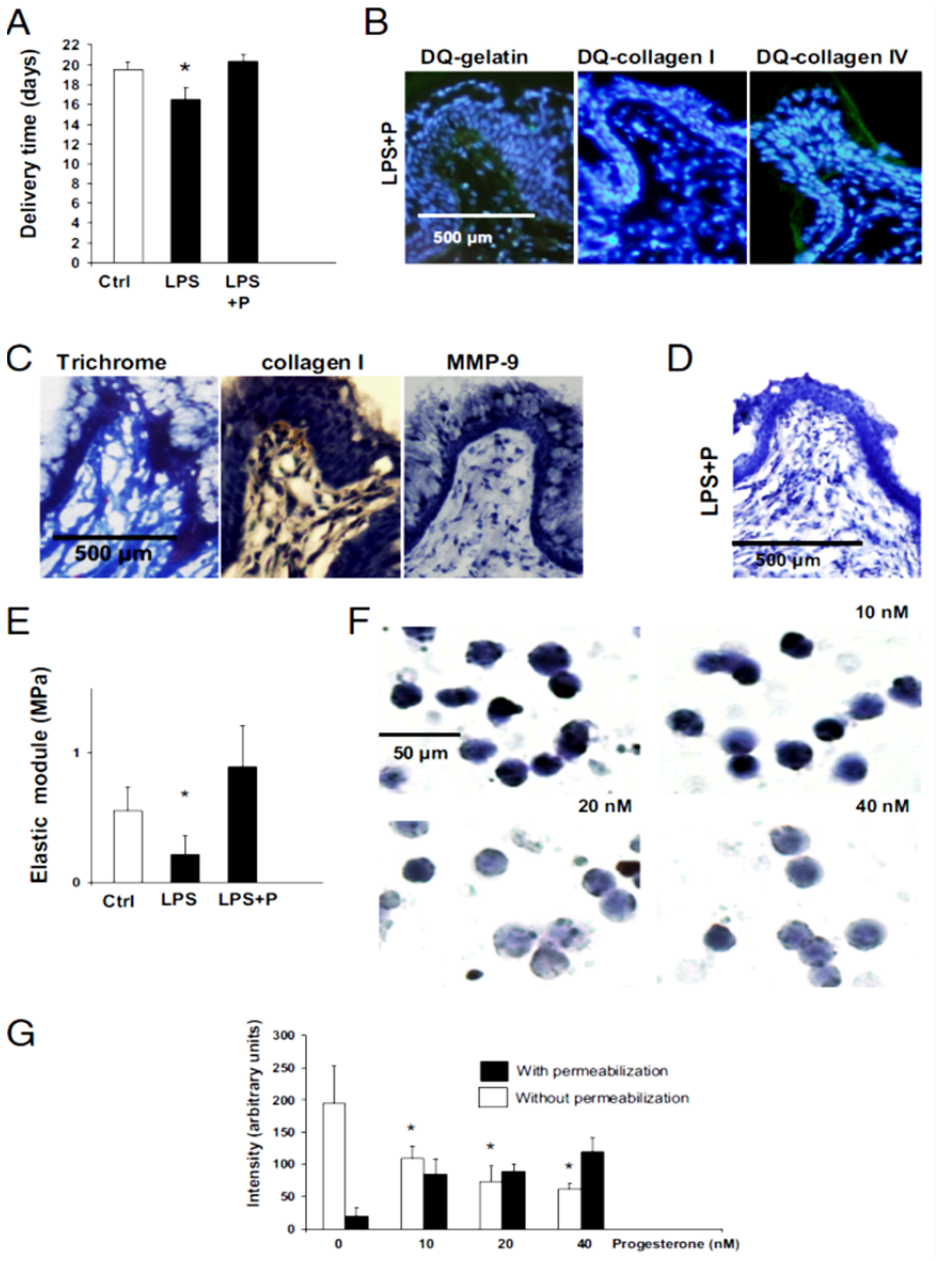

Figure 4. Progesterone (P) prevents preterm labor/delivery (PTD) in mice. A: Delivery time in control, LPS, and LPS plus P-treated mice (*n* = 5 to 7 mice/group). Significantly different from control, **P* < 0.05. Note that progesterone prevents PTD in LPS-treated mice. **B:** Histological studies. *In situ* zymography using DQ gelatin, DQ collagen I, and DQ collagen IV in the cervix of LPS plus P-treated mice. Decreased degradation of gelatin and collagen was observed in LPS plus P-treated mice when compared to LPS-treated mice (Figure 1E, middle panels). **C:** Staining for collagen in cervical tissue from LPS plus P-treated mice showed increased density of collagen fibers (**left** and **middle panels**) when compared to LPS-treated mice (Figure 1D, middle panels). Increased staining for MMP-9 observed in the cervix of LPS-treated mice (Figure 1F, upper middle panel) was not observed when mice were supplemented with progesterone (**right panel**). **D:** Negative staining for F4/80 in LPS plus P-treated mice. **E:** Biomechanical studies of the cervix. The elastic modulus (measure of the tissue stiffness) was decreased in the cervical tissue of LPS-treated mice when compared to control mice. Decreased elastic module was not observed in LPS-treated mice treated with P, indicating an increase in the stiffness of the cervical tissue in these mice. Elastic module values in LPS plus P-treated mice were comparable to age-matched control untreated mice (*n* = 3 to 4 mice/group). Significantly different from control mice (Ctrl), **P* < 0.05. **F:** Immunocytochemical detection of C5aR on murine cultured macrophages incubated without P and increasing doses of progesterone: 10 mmol/L, 20 mmol/L, and 40 mmol/L. **G:** Computerized quantification of C5aR staining on macrophages. Decreased staining for C5aR was observed in macrophages incubated with increasing doses of progesterone (white bars). Increased staining was observed in permeabilized cells (black bars) suggesting that C5aR is localized intracellularly in macrophages incubated with progesterone. Significantly different from control, **P* < 0.05.

caproate can reduce the rate of PTD in patients[22-31] and because progesterone inhibits the release of MMP-9 from macrophages in our mouse model, we investigated if progesterone prevented PTD in LPS-treated mice. Pretreatment with progesterone completely reduced the incidence of inflammation-induced PTD in LPS-treated mice (Figure 4A). Mice treated with progesterone and LPS delivered at a time not different from control untreated mice (Figure 4A). The cervical changes induced by LPS were not observed in mice pretreated with progesterone. Neither increased MMP activity (Figure 4B) nor increased collagen degradation (Figure 4C) was observed in LPS-treated mice pretreated with progesterone. In addition, progesterone prevented MMP-9 increase in cervical tissue in LPS-treated mice (Figure 4C). Interestingly, diminished macrophages infiltration was observed in the cervix of LPS plus progesterone-treated mice (Figure 4D), suggesting an anti-inflammatory effect of progesterone. Biomechanical studies performed in cervical tissue from LPS plus progesterone-treated mice showed increased elastic module values (increased stiffness) when compared to mice treated only with LPS (Figure 4E), suggesting that progesterone prevents inflammation-induced cervical remodeling in LPS-treated mice.

Progesterone Diminishes the Expression of C5aR in the Surface of Macrophages

Progesterone prevented inflammation-induced cervical remodeling and PTD in LPS-treated mice. Thus, progesterone would need to have anti-inflammatory effects to be able to effectively reduce the incidence of PTD. We hypothesized that the anti-inflammatory effect of progesterone observed in our studies is due to the down-regulation of C5aR on macrophages. Decreased staining for C5aR was observed in macrophages incubated with progesterone (Figure 4F). The amount of C5aR expressed on the surface of macrophages diminished with increasing doses of

progesterone (Figure 4F). Decreased expression of C5aR was not associated with decreased synthesis of C5aR. RT-PCR studies showed comparable levels of C5aR message in macrophages incubated with or without progesterone (false discovery rate < 0.1, fold change > 1.5). When macrophages were permeabilized before staining for C5aR, positive staining for C5aR in the cytoplasm was observed (Figure 4G). A positive correlation was observed between cytoplasmic staining for C5aR and increasing doses of progesterone (Figure 4G). This data suggests that progesterone induces the internalization of C5aR and thus diminishes the release of MMP-9, preventing cervical remodeling and PTD.

Discussion

We have shown by using two mouse models of preterm delivery induced by inflammation that complement activation plays an essential and causative role in the cervical structural changes that leads to preterm parturition. These studies identified complement split product C5a and macrophages as crucial mediators in the preterm birth.

In both mouse models of PTD, increased macrophages infiltration was observed in the cervical tissue, confirming the role of inflammation in PTD induced by LPS or RU486. The PTD model induced by RU486 was originally described as a model induced by progesterone withdrawal,[36] but it is now known that recruitment of immune cells that remodel the cervix is a crucial feature in the mechanism responsible for preterm birth in this model.[9,10] Our results showing increased cervical macrophages infiltration and increased C3a and C5a plasma levels clearly indicate that inflammation occurs in RU486 and LPS–treated mice that deliver preterm.

Collagen is the main component of the cervical stroma. These proteins are rigid

and nonextensible, and thus are responsible for the mechanical properties of the cervix. Throughout pregnancy collagenases slowly remodel (so–called softening and ripening) the collagen matrix in preparation for labor and delivery. In both models of PTD, extensive collagen degradation and decreased cervical stiffness occur, suggesting that these cervical changes are responsible for early delivery induced by inflammation.

Using histochemical and zymography techniques, we identified MMP–9 as a crucial metalloproteinase involved in the premature cervical remodeling observed in LPS– and RU486–treated mice. MMP–9 not only degrades gelatin, it is also a collagenase with the capacity to cleave collagen I, III, and IV.[39] Degradation of collagen fibers and disorganized collagen packing with greater spacing between fibers was observed in the cervix in LPS- and RU486-treated mice antepartum. By digesting collagen and gelatin, MMP-9 weakens the collagen matrix and thus the cervix dilates and PTD occurs.

We hypothesized that inflammation caused by LPS or RU486 creates pathological changes that accelerate the normal progress of events that lead to delivery. Thus, we investigated if inflammatory cells play a role in the cervical changes that lead to PTD induced in LPS- or RU486-treated mice. We found increased macrophages, but not neutrophils, infiltrating the cervical tissue in mice that deliver preterm. Depletion of macrophages abrogated cervical ripening and rescued pregnancies to term in LPS-treated mice, confirming the crucial role of macrophages in this model of PTD.

Complement activation has been related with many inflammation-mediated pregnancy disorders in mice and humans.[12,43-46] Increased C3a and C5a plasma levels were observed in the antepartum in mice treated with LPS or RU486 that deliver preterm. C5a is a potent chemotactic factor and activator of macrophages. Indeed, we

found that macrophages release MMP-9 in response to C5a and thus are an important source of MMPs.

In vivo studies confirmed the role of C5a and C5aR in the premature cervical remodeling and delivery in LPS- or RU486-treated mice. LPS- or RU486-treated, *C5aR−/−* mice showed collagen fibers densely packed and low metalloproteinase activity in the cervix. All these features are consistent with nondistensible cervixes and pregnancies that will go to term. Indeed, PTD was not observed in LPS- or RU4786-treated *C5aR−/−* mice. We also need to consider the possible contribution of cervical fibroblasts to the release of MMPs and the onset of PTD.[47] The absence of C5aR mRNA described in human cervical cancer TC-1 cells,[48] suggests that cervical cells do not express C5aR. However, cervical fibroblasts can also amplify MMPs release by a complement independent pathway.

Collectively our study demonstrates that LPS ascends from the vagina to the cervix, where the complement cascade is activated. C3b is deposited on the cervical epithelium and complement split product C5a is generated. C5a attracts and activated macrophages to the cervical stroma. In response to C5a, macrophages release MMP-9

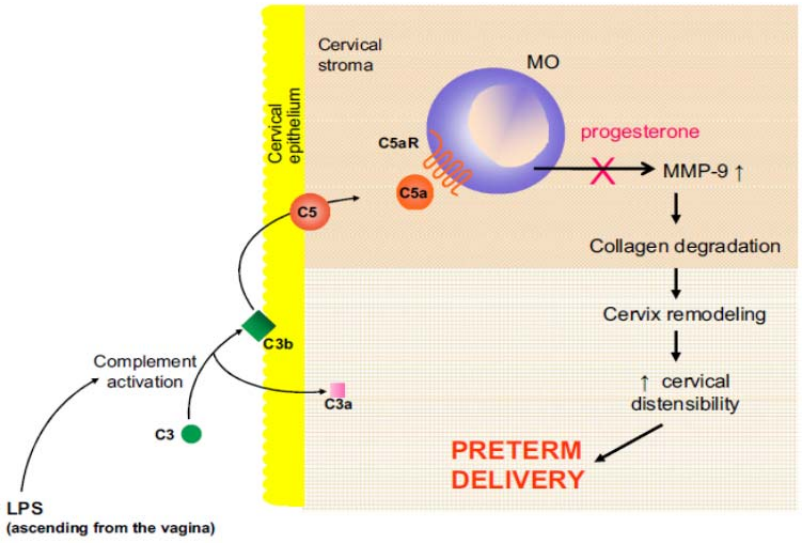

Figure 5. Proposed mechanism for LPS-induced cervical remodeling and preterm birth. LPS ascends from the vagina to the cervix, where the complement cascade is activated. C3b is deposited on the cervical epithelium and complement split product C5a is generated. C5a attracts and activated macrophages to the cervical stroma. In response to C5a macrophages release matrix metalloproteinase (MMP)-9 that digests collagen leading to cervical remodeling, increased cervical distensibility, and preterm birth.

that digests collagen leading to cervical remodeling and preterm birth (Figure 5).

Based on the beneficial effects of progesterone in some human studies[22-31] and on the anti-inflammatory effects of progesterone,[33,49] we investigated if progesterone supplementation could prevent cervical remodeling and preterm birth in mice treated with LPS. Our studies demonstrated that progesterone pretreatment abrogated the cervical changes that lead to PTD in LPS-treated mice. Neither increased MMPs activity nor collagen degradation and loss of tensile strength was observed in the cervixes of LPS-treated mice that received progesterone. In addition, progesterone inhibited C5a-induced release of MMP-9 from macrophages *in vitro*. C5aR expression was reduced on the surface of macrophages incubated with progesterone in comparison to those incubated with only media suggesting that decreased C5aR might contribute to the inhibitory effect of progesterone on MMPs release. However, when macrophages incubated with progesterone were permeabilized before staining, C5aR was clearly detectable in the cytoplasm of macrophages. This data suggests that C5aR is internalized in macrophages incubated with progesterone. Internalization of C5aR (as many other G-coupled receptors) has been described in neutrophils and macrophages in mice and may account for diminished activity observed in these cells.[50,51] Internalization of C5aR by progesterone can also explain the diminished macrophage infiltration observed in LPS plus P-treated mice. This observation is supported by the findings of Timmons and Mahendroo.[10]

The protective effects of progesterone in PTD can also be caused by the inhibitory effects of progesterone on MMP-9 synthesis. It has been reported that progesterone inhibits MMP-9 synthesis in rabbit uterine cervix[42] and ischemic brains in rats.[52] However, we did not find decreased MMP-9 mRNA in macrophages incubated

with progesterone, but we found decreased release of MMP-9. Internalization of C5aR on macrophages and consequent diminished cell activation by C5a may explain the diminished release of MMP-9 from macrophages incubated with progesterone. The fact that progesterone prevents premature birth in the mouse model of LPS-induced PTD that showed no diminution in serum progesterone levels suggests that progesterone is acting as an anti-inflammatory molecule. Moreover, progesterone diminished macrophages infiltration in cervical tissue in LPS-treated mice. Several studies support the concept that progesterone has anti-inflammatory properties.[49,53] Progesterone suppresses estrogen-induced C3 expression in the mouse oviducts and uterus.[54] Another report showed that progesterone suppresses the complement-mediated attack to allogenic chick embryos.[55] Thus, we also need to consider that progesterone may prevent PTD in LPS-induced PTD by modulating the expression and/or activation of complement components.

Our studies showed the effectiveness of progesterone in preventing LPS-induced cervical remodeling. However, we also need to consider that progesterone may also contribute to the prevention of PTD by promoting uterine quiescence. Several studies demonstrated that progesterone promotes myometrial relaxation.[56-58]

Our finding that the absence of C5aR prevents PTD in the mouse models may have important therapeutic implications. Blocking the complement cascade at C5a and CaR interaction inhibits mediators and effectors of cervical remodeling and prevents PTD, suggesting that complement might be a good target for therapy in women with PTD. One of the few current treatments for women at risk of preterm labor is progesterone, which can diminish the expression of C5aR on the surface of macrophages and thus prevent inflammation, the release of MMPs, and cervical

remodeling, reinforcing the idea that the complement system is a promising target for therapy in this serious pregnancy complication. Complement inhibitors are now being tested in patients with inflammatory, ischemic, and autoimmune diseases. Identifying complement-related markers that predict high risk for PTD will allow us design interventions that prevent or arrest this pathological process, which compromises the survival of the neonates born prematurely.

CHAPTER 2

COMPARISON OF THE MECHANISMS RESPONSIBLE FOR CERVICAL REMODELING IN PRETERM AND TERM LABOR

Introduction

The process of parturition has been extensively studied. However, the mechanisms responsible for the onset and progression of labor are multifactorial and not completely understood.[1,59,60] Understanding the mediators and effectors of labor could have enormous benefit, allowing potential interventions for efficient induction of labor or conversely in postponing unwanted labor. Identification of targets to regulate or terminate labor is of particular importance in the treatment and prevention of preterm labor, which continues to be a major public health issue and the number one cause of neonatal death. Among the important events contributing to labor is the ripening/ remodeling of the cervix.[2,38,61] Changes in the cervical structure soften and dilate the cervix allowing the passage of the fetus forced by rhythmic myometrial contractions. Chapter 1 reveals how we demonstrated that complement activation plays a crucial role in the cervical remodeling process that leads to preterm labor. Using a mouse model of PTD induced by LPS we found that complement split product C5a attracts and activates macrophages to the cervix. In response C5a, macrophages release metalloproteinases (MMPs) that degrade collagen, increasing the cervix distensibility and leading to PTD in mice.[62]

The aim of this study is to characterize the term cervical ripening process and compare it with preterm. Knowing that complement activation plays a causative role in PTD in mice, we decided to study if complement activation also plays a role in cervical remodeling at term. If complement activation is not involved in the physiological

process that leads to term delivery we will then have identified a possible specific and selective target to prevent PTD and thus improve neonatal health.

Materials and Methods

Ethics Statement

As noted in chapter 1, our animal studies were performed following the Russell and Burch Three R's (Replacement, Reduction and Refinement) concept to minimize animal use and pain or distress while still achieving the critical scientific objectives. All the experiments performed were conducted in accordance with the National Institute of Health guidelines on laboratory animals and with approval from the Wayne State University (protocol A 09-08-09) and York College CUNY committee on Animal Use and Care (protocol R-3-2009).

Animals

C57BL/6 timed-pregnant mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were shipped on day 10 to 12 after mating. Animals were acclimated in the animal facility for 3 to 5 days before use in these experiments.

Cervical remodeling was studied in mice at term and during preterm labor. A mouse model of PTD which resembles most clinical scenarios in that localized inflammation occurs without systemic maternal illness was used. In this model, mice were treated with LPS (E. coli serotype 055:B5 (250 μg/mouse, intravaginally)) on day 15 of pregnancy. Most of the pregnant females (94.7%) delivered within 24–36 hours of LPS administration.[62] No maternal morbidity or mortality was observed in this model.

Pregnant mice treated with LPS were euthanized antepartum (between 12–18 hours after treatment) or during delivery (intrapartum). Delivery was considered preterm if it occurred within 48 hours after LPS administration (before gestational day 17). In our

animal facilities, term delivery occurs between day 20 and day 21 of pregnancy. Control mice were euthanized antepartum (day 17, 18 and 19) or intrapartum (by direct observation after the passage of 1 or 2 pups). Blood samples and cervical tissue were collected.

Serum complement activation products and progesterone levels

To determine the role of complement activation in term and PTD, generation of complement split products C3a and C5a were measured by measuring the more stable metabolites C3adesArg and C5adesArg in serum. C3a(desArg) and C5a(desARg) result from the removal of the C-terminal arginine by ubiquitous carboxypeptidases and have longer half time than C3a and C5a. C3adesArg in serum was measured by sandwich ELISA as previously described in Chapter 1 using rat anti-mouse C3a and biotin rat anti-mouse C3a. Both antibodies were purchased from BD Biosciences Pharmingen, CA. A Standard curve was performed using purified mouse C3a protein (native) (BD Biosciences Pharmingen). C5adesArg was also measured by sandwich ELISA using rat anti-mouse C5a and biotin rat anti-mouse C5a. Both antibodies and mouse purified C5a protein for the standard curve were purchased from BD Biosciences.

Progesterone levels were measured by competitive enzyme immune assay (Cayman Chemicals, Ann Arbor, MI).

Immunohistochemistry and immunocytochemistry

Cervical tissues isolated during antepartum and intrapartum were frozen in O.C.T. compound, and cut into 10 μm sections. Sections were stained for C3 with rabbit anti-mouse C3 (LifeSpan Biosciences, Seattle, WA), neutrophils were stained with rat anti-mouse granulocyte RB6-8C5 mAb (BD Biosciences Pharmingen) and

macrophages with F4/80 (Novus Biologicals, Inc, Littleton,CO). MMP-2 and MMP-9 were detected in frozen cervical sections using rabbit polyclonal anti-mouse MMP-2 and MMP-9 antibodies (Abcam, Cambridge, MA). Collagen I distribution was determined using Masson's Trichrome staining and by immunohistochemistry using a polyclonal antibody against collagen type I (ACRIS Antibodies, GmbH, Herford, Germany). HRP-labeled specific secondary antibodies and DAB as substrate were used to develop the respective reactions.

In situ zymography

Metalloproteinases MMP-2 and MMP-9 activity against collagen I and IV and gelatin were measured by *in situ* zymography as previously described in chapter 1.[37] Briefly, 10-µm-cervix sections were washed in PBS and then incubated for 4 hours with different MMPs DQ substrates. DQ-gelatin, DQ-collagen I and DQ collagen IV (Invitrogen, Carlsbad, CA). The enzyme-driven hydrolysis of these substrates results in an increase in fluorescence signal. Increased fluorescence indicates increased gelatin and collagen degradation by MMPs. In parallel, control sections were preincubated with buffer containing the MMP inhibitor EDTA to indicate the contribution of MMPs. The reaction was stopped by a 10 minutes incubation in 4% paraformaldehyde-PBS. Finally, mounting medium supplemented with DAPI (Vector Laboratories, Burlingame, CA) was applied. Sections (4–6 tissue sections/group) were observed under a fluorescence microscope Nikon Eclipse 50i (Nikon Inc, Melville, NY) and photographs were taken using a Nikon DigiSight Color Digital Camera System and NIS-Elements Research Imaging Software.

Human cervical epithelial cells

A cell line from normal human endocervical epithelia immortalized by expression

of human papillomavirus (Endl/E6E7) (ATCC Manssas, VA) was used in *in vitro* studies.[63] The morphology of these immortalized cells corresponds to glandular tall columnar epithelial cervical cells and closely resembles that of the tissue of origin and primary cultures. Thus, End1/E6E7 cells constitute a reproducible in vitro model to study the role of these epithelial cells in cervical ripening. End1 cells were cultured as previously described.[63] The expression of C5aR on End-1cells was studied by immunohistochemistry using anti-human C5aR antibodies (Cell Sciences, Canton, MA). MMP-2, MMP-9 and progesterone receptors (PR) expression in End-1 cells was also evaluated by RTPCR. Aliquots of End1 cells were incubated for 16 h with progesterone (1 mg/ml) and synthesis of MMP-2 and MMP-9 was studied by RT-PCR.

Aliquots of End1/EGE7 cells were cultured on cell chamber slides incubated with and without progesterone (1 mg/ml) for 16 h and then subjected to *in situ* zymography using DQ-gelatin and DQ-collagen I as previously described. Immunohistochemistry was performed to detect MMP-2 and MMP-9 using rabbit polyclonal anti-mouse MMP-2 and MMP-9 antibodies (Abcam, Cambridge, MA) followed by a FITC-labelled anti-rabbit antibody (Abcam, Cambridge, MA).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

To determine whether End1 synthesize MMP-2, MMP-9 and PR, qRT-PCR was performed. RNA was harvested from End1 cells with RNeasy Mini Kit (Qiagen, Valencia, CA) and 1 µg of total RNA was reverse transcribed. Primer sequences mouse GAPDH, human MMP-2, MMP-9 and PR were obtained from Applied Byosystems (Foster City, CA). Relative expression was normalized for levels of GAPDH. Relative quantification of MMP-2 and MMP-9 between End-1 cells incubated with and without progesterone gene expression was performed using 2^{-DDCT} data analysis. The

comparison was performed in pairs using the same target gene and the same End-1 samples. CT = Threshold value; DCT = average target CT – average GAPDH CT; DDCT (for the same target gene) = average DCT End-1 incubated with progesterone- average DCT End-1 incubated without progesterone. DDCT = 0 when the End-1 of interest are used as calibrator. 2^{-DDCT} = normalized target gene amount (MMP-2 or MMP-9) relative to target gene amount in End-1 cells incubated without progesterone. * $p<0.05$, n = 4.

Results

Serum progesterone levels in term and preterm delivery

In most mammalian species aside from the great apes, labor is initiated by a decrease in circulating progesterone levels (progesterone withdrawal).[64] Indeed, we found that in mice, serum progesterone levels drop dramatically intrapartum when compared to antepartum levels (Figure 6). On the other hand, mice treated with LPS that deliver preterm do not show a diminution in progesterone levels compared to antepartum levels. Progesterone levels in antepartum were comparable to intrapartum values in LPS-treated mice (Figure 6), suggesting that progesterone withdrawal is not involved in preterm parturition in LPS-treated mice.

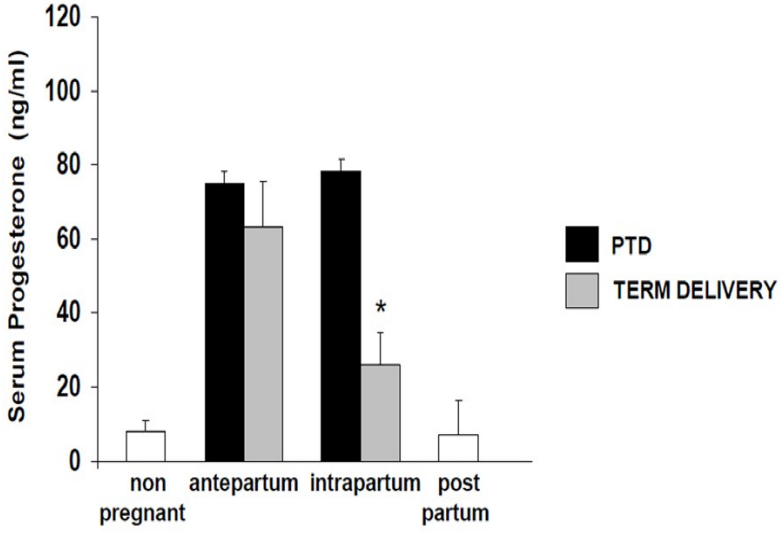

Figure 6. Serum progesterone levels in PTD and term delivery. Antepartum LPS serum samples were collected at day 15 of pregnancy, intrapartum LPS samples at days 16 and 17, antepartum term samples at days 17, 18 and 19 and intrapartum term at days 20 and 21. Note that progesterone levels drop dramatically near parturition time (days 20 and 21) in control mice at term while LPS-treated mice that delivered preterm did not show changes in progesterone levels. * Different from antepartum, $P<0.05$. 3 to 5 samples were analyzed at each time point.

Complement activation in term and preterm delivery

Complement activation involves the cleavage of C3 into C3b that deposits on the tissue, and C3a that can be measured in systemic circulation. Increased C3 deposition was observed in the cervix of LPS-treated mice with antepartum and intrapartum (Figure 7 A). Prompted by the findings that complement activation triggers cervical remodeling and preterm labor (Figure 7A), we investigated if complement activation also

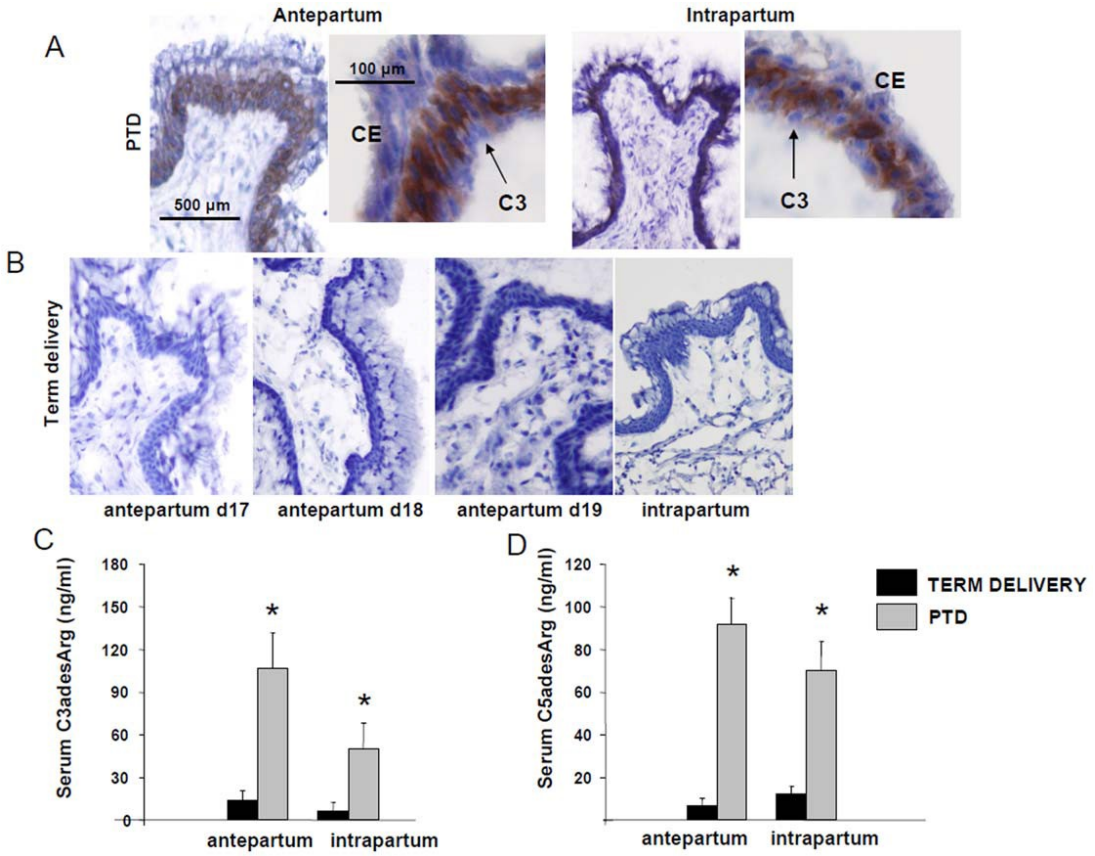

Figure 7. Complement activation in PTD and term delivery. A- Complement C3 deposition in the cervix of mice that delivered preterm antepartum and intrapartum. Black arrows point to complement deposition in the cervical columnar l epithelium (CE). Original magnification is ×20. **B-** Complement C3 deposition in the cervix of mice that deliver at term. C3 deposition was not observed antepratum (days 17, 18 and 19) or intrapartum. Original magnification is ×20. For C3 determination, 4 slides per animal were stained and 6–8 animals were studied in each group. **C–D** C3adesArg and C5adesArg levels in serum from mice with PTD and term delivery collected antepartum and intrapartum. Antepartum LPS serum samples were collected at day 15 of pregnancy, intrapartum LPS samples at days 16 and 17, antepartum term samples at days 17, 18 and 19 and intrapartum term at days 20 and 21. * Statistically different from control, $p<0.05$. n = 5–6 mice/group.

contributes to term labor. Surprisingly, no C3 cervical deposition was observed in mice that delivered at term (Figure 7B). Negative staining for C3b was observed in cervical samples collected antepartum (days 17, 18 and 19) or intrapartum (Figure 7B). In addition, mice that delivered at term showed no increase in serum C3a and C5a levels, neither antepartum nor intrapartum (Figure 7C and 7D). C3a and C5a levels in mice that delivered at term were significantly lower than those observed in mice that delivered preterm (Figure 7C and 7D). The absence of complement split products at term labor suggests that complement activation is not involved in the physiological process that leads to term parturition.

Inflammatory cells in term and preterm delivery

In Chapter 1 we previously demonstrated that inflammation plays a crucial role the cervical remodeling that leads to PTD. To determine whether inflammatory cells are also required for the cervical ripening at term, we performed immunohistochemical studies in cervical samples harvested antepartum and intrapartum in control untreated mice. The presence of macrophages was studied using antibodies against F4/80 and neutrophils were identified with antibodies anti-Gr1. Increased macrophages were observed antepartum and intrapartum in the cervix of mice that deliver preterm (Figure 8). In contrast, no macrophages were observed in samples collected antepartum and intrapartum at term (Figure 8).

The absence of inflammatory cells indicates that the cervical remodeling that leads to term labor is not dependent on leukocytes. That inflammatory cells do not participate in the cervical changes that contribute to term labor constitute a significant difference between the term and preterm mechanisms of labor.

Staining with anti-Gr1 showed that neutrophils are not present either in the cervix

of mice at term or preterm (Figure 8).

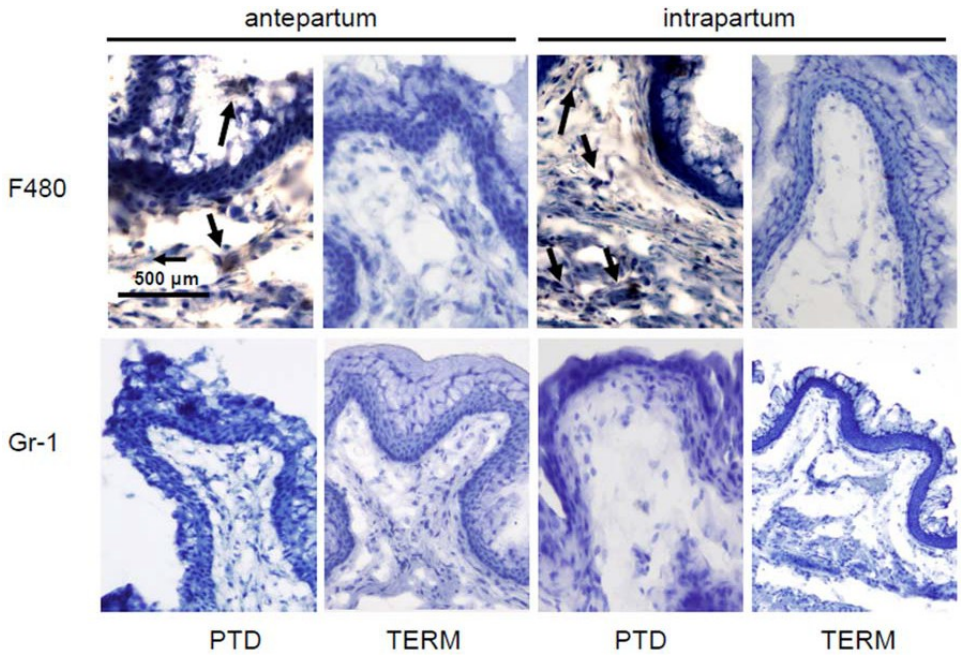

Figure 8. Inflammatory cells in cervical tissue from mice that deliver preterm and at term. F4/80 staining in cervical tissue. Increased macrophage infiltration **(black arrows)** was observed in the cervix of mice that deliver preterm antepartum and intrapartum. In contrast no macrophages were observed in the cervix of mice that deliver at term antepartum and intrapartum. Original magnification is ×20. B- Gr-1 staining in cervical tissue. No neutrophil infiltration was observed at preterm or term cervical samples. Original magnification is ×20. 4 slides per animal were stained and 6–8 animals were studied in each group.

MMPs synthesis in human cervical columnar epithelial cells End1

MMPs participate in cervical remodeling by degrading the network of collagen fibers and thus increase the distensibility required for delivery.[3,65] It has been extensively documented that inflammatory cells, macrophages and neutrophils, are a good source of MMPs. The absence of inflammatory cells in the cervical tissue at term leads as to investigate other possible sources of MMPs. In the absence of inflammatory cells, cervical columnar epithelial and stromal fibroblastic cells could be alternative sources of MMPs. To determine whether cervical columnar epithelial cells are capable of synthesizing MMPs, we performed qRT-PCR. MMP-2 and MMP-9 mRNA was found in End-1 cells (Figure 9B), indicating that cervical columnar epithelial cells can be a

source of MMPs in cervical remodeling at term. *In situ* zymography studies show that End-1 cells produce and release proteases that digest gelatin and collagen I (Figure 9C). These active proteases were identified as MMP-2 and MMP-9 by immunohistochemistry (Figure 9C).

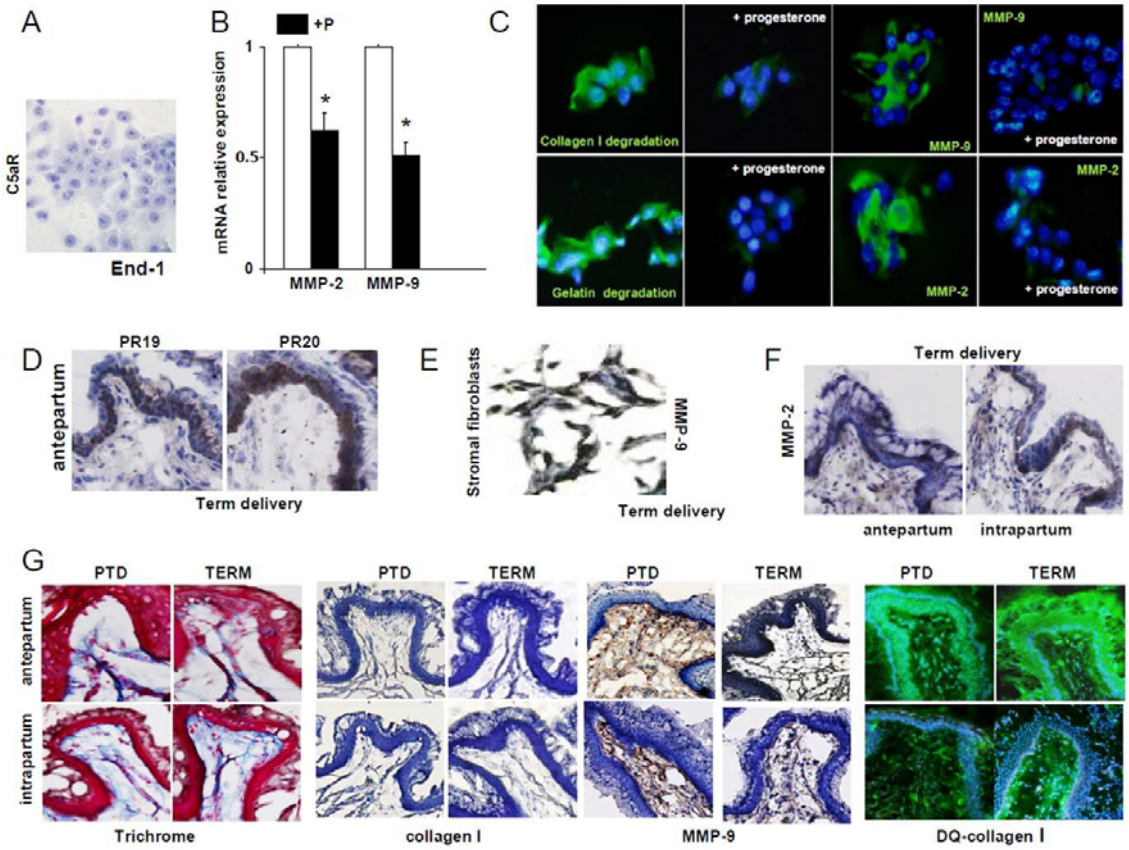

Figure 9. Cervical MMPs activity and collagen degradation at PTD and term delivery. A- Immunohistochemical determination of C5aR in End-1 cervical columnar epithelial cells. n = 4. **B–** Relative quantification of MMP-2 and MMP-9 between End-1 cells incubated with and without progesterone. * $p<0.05$, n = 4 mice/group. Note that in End-1 cells incubated with progesterone a ~50% reduction in MMP-2 and MMP-9 mRNA was observed when compared to untreated cells. **C-** In situ zymography using substrates DQ-gelatin and DQ-collagen I and immunohistochemical detection of MMP-2 and MM9-9 en End-1 cells incubated with and without progesterone. The green fluorescence indicate the substrate has been digested. MMP-2 and MMP-9 were detected by IHC using specific antibodies followed by FITC-labeled secondary antibodies. D- Immunohistochemical detection of progesterone receptors PR-19 and PR-20 in the cervix of mice during antepartum at term. 4 slides per animal were stained and 6–8 animals were studied in each group. **E-** Trichrome staining, staining for collagen I and for MMP-9 and in situ zymography using DQ-collagen I in cervical tissue harvested antepartum and prepartum from mice that deliver preterm and at term. 3–4 slides per animal and 5 to 6 mice/group were used for immunohistochemical studies. Original magnification is ×20. **F-** Immunohistochemical determination of MMP-9 in cervical stromal fibroblasts at term. 4 slides per animal were stained and 6–8 animals were studied in each group. **G-** Immunohistochemical determination of MMP-2 in cervical columnar epithelial and stromal cells at term during antepartum and intrapartum. 4 slides per animal were stained and 6–8 animals were studied in each group.

Next we investigated the presence of C5aR in End-1 cells. C5aR could not be detected on End-1 cells by immunohistochemistry (Figure 9A). The absence of C5aR in End-1 cells suggests that cervical columnar epithelial cells are not cellular effectors in PTD, a complement-dependent process. Indeed, in chapter 1 we describe that macrophages rich in C5aR are the main source of MMPs in PTD.

In addition, we found that End-1 cells express progesterone receptor (PR) at the mRNA level. Interestingly, progesterone that inhibited MMP release from macrophages in PTD inhibits the synthesis of MMP-2 and MMP-9 in these cells (Figure 9B). In agreement with these results, we were able to detect progesterone receptors (PR) 19 and 20 in mouse cervical epithelial cells by immunohistochemistry. As shown in Figure 9D, positive staining for PR-19 and PR-20 was observed in the cervical columnar epithelium at term.

We also explored the possibility that cervical fibroblastic cells express MMPs. In accordance to our hypothesis, we found positive staining for MMP-9 in the fibroblasts stroma harvested antepartum in mice that deliver at term (Figure 9E). Thus, cervical stromal fibroblastic cells can be another source of MMPs leading to cervical remodeling and parturition at term. In addition, we measured MMP-2, another proteolytic enzyme involved in cervical remodeling. We detected MMP-2 in the epithelium and stroma antepartum and intrapartum at term (Figure 9F). We were not able to detect MMP-2 expression in the cervical tissue of mice that deliver preterm (data not shown).

Cervical MMPs and collagen degradation in term and preterm delivery

MMPs degrade collagen in the cervix and prepare it for dilation and delivery.[3,65] Regardless the different sources of MMPs in preterm and term, remodeling of the cervix and massive collagen degradation was observed antepartum and intrapartum in both

term and preterm delivery (Figure 9G). Masson's trichrome (TC) staining revealed a loose array of disordered collagen fibers in the cervix of mice that deliver preterm and term (Figure 9G). Immunohistochemical studies with specific antibodies against mouse collagen I showed a similar distribution pattern of collagen (Figure 9G). Collagen packing was disorganized, collagen fibers looked thinner and greater spacing between fibers were observed both antepartum and intrapartum in mice that delivered at term and preterm (Figure 9G).

Decreased density in collagen fibers was associated with increased expression of MMP-9 (Figure 9G). *In situ* zymography using DQ collagen I as substrate revealed an increase in collagenolytic activity in the cervix of mice that deliver at term and preterm (Figure 9G).

Discussion

Is cervical remodeling in PTD caused by the same mechanism/s that cause/s cervical remodeling at term, but are accelerated in time? This question has been pondered by obstetricians seeking for strategies to prevent PTD for many years. To answer this question we investigated the initiators and cellular effectors in a mouse model of preterm delivery and in control mice that delivered at term. In Chapter 1 we described that complement activation plays a causative role in PTD in mice. Thus, we sought to investigate whether complement activation also plays a role in cervical remodeling at term. Here we found that complement activation is not involved in the physiological process that leads to term delivery suggesting that we identified a possible specific and selective target to prevent PTD. In addition, serum complement C3a and C5a levels, that increased during PTD did not increase in mice that deliver at term. That C3a and C5a increase only during PTD suggests that they can be potential

biomarkers. This is in agreement with several clinical studies that demonstrate a potential role of complement split products as biomarkers of PTD.[15,16,21]

Recent studies in mice suggested that the mechanisms of cervical remodeling in preterm birth is different from normal ripening at term.[66,67] In one of these studies the expression of proinflammatory genes was upregulated in preterm birth compared to term.[66] The other study demonstrated that cervical ripening preterm can be initiated by more than one mechanism and it is not necessarily an acceleration of the physiological process at term.[67]

Our data suggest that the cervical ripening at term is a non-leukocyte dependent process. We did not find inflammatory cells in the cervix of mice with term parturition in contrast to mice that delivered preterm. Several animal studies support our observation that cervical remodeling at term is leukocyte-independent. Steroid 5alpha-reductase type 1 null mice (Srd5a1−/−) that do not recruit inflammatory cells in the cervix give birth normally suggesting that inflammatory cells are not required for term parturition.[10] In addition, granulocyte depletion with monoclonal antibodies LyG (Gr1) before birth has no effect on the timing or outcome of parturition in mice.[10] A human study emphasizes the lack of correlation between inflammation and cervical remodeling at term. In this study, the authors reported that inflammation-related genes did not emerge as differentially expressed with cervical ripening.[68] Even though several studies in animals suggest that inflammatory cells do not participate in cervical ripening at term, some human studies suggest that inflammatory cells do participate in the cervical changes in term parturition.[69,70] This discrepancy might be caused by the fact that when the biopsies are obtained; intrapartum may overlap with postpartum and the cervical repair process might have already started. Indeed many studies demonstrate the crucial role

of inflammatory cells in the cervical tissue repair after delivery.[10]

We demonstrated in Chapter 1 that complement component C5a recruits and activates macrophages in cervical tissue leading to cervical ripening and PTD in LPS-treated mice. The absence of complement activation and generation of C5a in cervical tissue at term might explain the absence of inflammatory cells. Despite the absence of complement activation and inflammatory cells at term, our data demonstrate the presence of MMPs and concomitant collagen degradation in the cervix of mice that deliver at term and preterm. Increased expression of MMP-9, disorganized collagen packing, thinner collagen fibers and greater spacing between fibers were observed in both term and preterm. This suggests the existence of a common downstream pathway shared by term and preterm cervical remodeling.

The identification of this common mechanism raises the question as to which cells are responsible for MMP release during cervical ripening at term. It has been established that cervical fibroblasts obtained from pregnant women are capable of releasing MMPs in culture.[71] Other studies also showed that human cervical fibroblasts can also synthesize MMPs.[42,72] In addition, Imada and collaborators demonstrated that cervical fibroblasts from rabbits synthesize MMP-9 in culture.[42] Indeed, we found positive staining for MMP-9 in cervical fibroblastic cells in the stroma of control mice at term.

Staining for MMP-9 and robust activity against collagen I was also observed in the cervical columnar epithelium in mice at term. Thus, we performed *in vitro* studies using End-1 cells[63] (human columnar epithelial cells) to corroborate that these cells can indeed synthesize and express MMPs. qRT-PCR studies revealed the presence of MMP-2 and MMP-9 mRNA and in situ zymography and immunohistochemistry studies

demonstrate the presence of active MMP-2 and MMP-9 in End-1 cells, suggesting that columnar epithelial cells can be the source of MMPs responsible for cervical remodeling that leads to parturition at term.

We also need to consider that cytokines secreted by the myometrium, placenta and fetal membranes at term contribute to the release of MMPs in the cervix in the absence of inflammatory cells.[73,74] Thus, it is possible that inflammatory mediators secreted by neutrophils infiltrating the fetomaternal interface reach the cervical stroma and promote MMPs release.

It has been described that progesterone inhibits the release of MMPs and collagenolysis.[75,76] Interestingly, we found that progesterone inhibits the synthesis and expression of MMP-2 and MMP-9 in End-1 cells. That progesterone inhibits MMPs release suggests that a diminution in progesterone levels might trigger the release of MMPs and induce cervical ripening at term. Indeed, we found that progesterone levels drop dramatically in mice around parturition time at term, in contrast to mice that deliver preterm. This observation suggests that progesterone withdrawal can trigger MMPs release and subsequent parturition at term as has been suggested.[64] We were not able to detect C5aR on End-1 cells, suggesting that complement activation that is responsible for the release of MMP-9 from macrophages in PTD is not involved in MMP release from cervical columnar epithelial cells. Thus, we propose that progesterone withdrawal triggers MMPs release and parturition at term. That progesterone receptors PR-19 and PR-20 are present in cervical epithelial cells reinforces this concept.

In Figure 10 we summarize the distinct and similar features that characterize cervical remodeling at preterm and term. 1) Preterm delivery is initiated by complement activation while complement activation is not required for term parturition. 2) Term

delivery was found to be non-leukocyte dependent while on the other hand PTD is mediated by macrophages. 3) Macrophages are the source of MMPs in PTD while cervical stromal fibroblasts and columnar epithelial cells seem to be responsible for the production of MMPs in cervical remodeling at term. 4) The trigger for MMPs release is C5a in PTD while our studies and other studies[64] suggested that progesterone withdrawal is the initiator of cervical ripening at term.

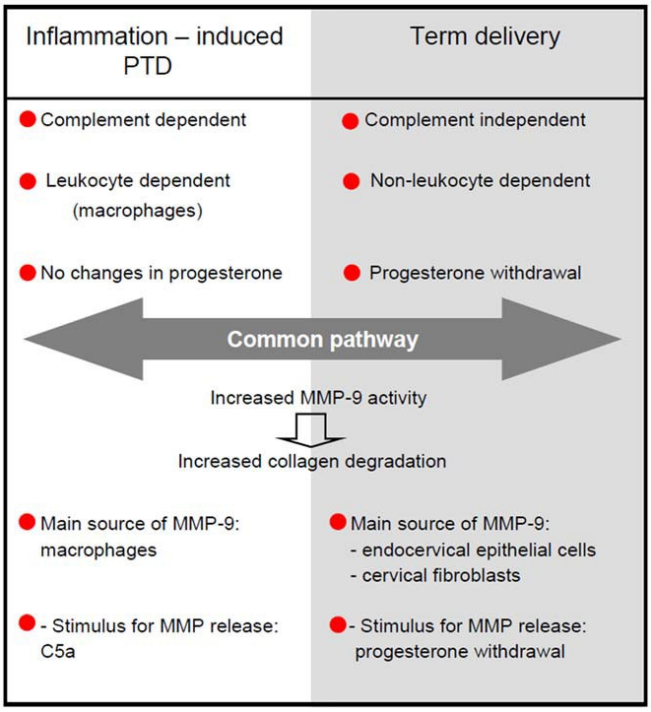

Figure 10. Distinct and similar features during preterm and term cervical remodeling. PTD is initiated by complement activation while complement activation is not required for term parturition. In addition term delivery was found to be non-leukocyte dependent while on the other hand preterm delivery is mediated by macrophages. A dramatic diminution in serum progesterone levels precedes parturition at term but no progesterone levels changes are observed in PTD. PTD and term delivery share a common dowsntream pathway characterized by increased MMPs release and increased collagen degradation. The sources of MMPs in PTD are macrophages while cervical stromal fibroblasts and columnar epithelial cells seem to be the source of MMPs at term. Anaphylotoxin C5a is the trigger for MMPs release in PTD while progesterone withdrawal seems to be the trigger at term. In conclusion, preterm and term cervical remodeling occur through the same mechanism but it is initiated by different triggers and effector cells.

Regardless the different triggers and cellular mediators, we identified a common downstream pathway that involves MMPs release and collagen degradation in both term and PTD.

In conclusion, preterm and term cervical remodeling occur through the same mechanism but they are initiated by different triggers and effector cells. Thus, we demonstrated that preterm birth is not an acceleration of the normal physiological cervical processes that lead to term parturition.

This is the first study to identify the complement system as a potential biomarker and possible specific and selective target for therapy in PTD.

APPENDIX A

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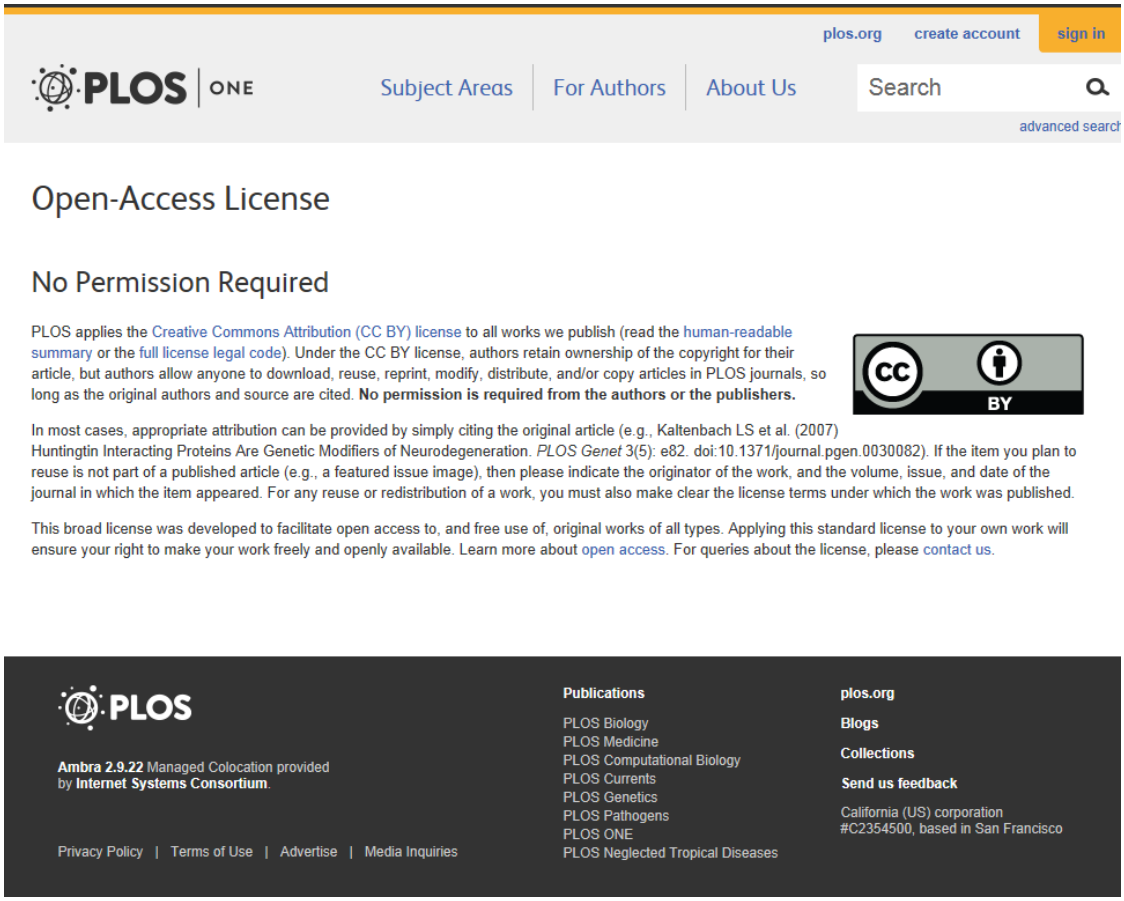
Gonzalez JM, Dong Z, Romero R, Girardi G. Cervical remodeling/ripening at term and preterm delivery: the same mechanism initiated by different mediators and different effector cells. ***PloS one*** 2011;6:e26877



Gonzalez JM, Franzke CW, Yang F, Romero R, Girardi G. Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. ***Amer. Journal of Pathology*** 2011;179:838-49

The American Journal of Pathology

Volume 179, Issue 2, August 2011, Pages 838–849

Regular article

Complement Activation Triggers Metalloproteinases Release Inducing Cervical Remodeling and Preterm Birth in Mice

Juan M. Gonzalez[*], Claus-Werner Franzke[†], Fengyuan Yang[‡], Roberto Romero[*], Guillermina Girardi[§]

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APPENDIX B

IACUC Protocol Approval Letter

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A 3310-01

PROTOCOL # A 09-07-09

Protocol Effective Period: October 23, 2009 – September 30, 2012

Year 2 Annual Review Date: October 1, 2010

TO: Dr. Sonia Hassan
Obstetrics and Gynecology
4802 Hutzel Hospital

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 09-07-09
"Molecular Mechanisms of Cervical Remodeling"

DATE: October 1, 2010

The Annual Review of your animal research protocol and any applicable grant applications has been conducted and approved by the Wayne State University Institutional Animal Care and Use Committee (IACUC). The species and number of animals approved for the duration of this protocol are listed below.

Species	Strain	Qty.	Cat.
MICE	CD1, timed pregnant, six weeks/40g	393	D
MICE	CD1, female, six weeks/20g.	20	D
MICE	CD1, male/female, pups and fetuses from timed pregnant dams	4716	C

Species Amendments	Strain	Qty.	Cat.
NONE			

Be advised that any change in the procedures used, a change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office of Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

REFERENCES

1. Iams JD, Berghella V. Care for women with prior preterm birth. American journal of obstetrics and gynecology 2010;203:89-100.
2. Word RA. Cervical function during pregnancy and parturition. In: F P, ed. Preterm Birth: Mechanisms, mediators, prediction, preventions and interventions. London: Informa Healthcare 2007:19-34.
3. Stygar D, Wang H, Vladic YS, Ekman G, Eriksson H, Sahlin L. Increased level of matrix metalloproteinases 2 and 9 in the ripening process of the human cervix. Biology of reproduction 2002;67:889-94.
4. Hamilton BE, Martin JA, Ventura SJ. Births: preliminary data for 2005. National vital statistics reports : from the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System 2006;55:1-18.
5. Steer P. The epidemiology of preterm labour. BJOG : an international journal of obstetrics and gynaecology 2005;112 Suppl 1:1-3.
6. Elovitz MA, Mrinalini C. Animal models of preterm birth. Trends in endocrinology and metabolism: TEM 2004;15:479-87.
7. Yoon BH, Romero R, Moon JB, et al. Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. American journal of obstetrics and gynecology 2001;185:1130-6.
8. Wei SQ, Fraser W, Luo ZC. Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review. Obstetrics and gynecology 2010;116:393-401.
9. Denison FC, Riley SC, Elliott CL, Kelly RW, Calder AA, Critchley HO. The effect

of mifepristone administration on leukocyte populations, matrix metalloproteinases and inflammatory mediators in the first trimester cervix. Molecular human reproduction 2000;6:541-8.

10. Timmons BC, Mahendroo MS. Timing of neutrophil activation and expression of proinflammatory markers do not support a role for neutrophils in cervical ripening in the mouse. Biology of reproduction 2006;74:236-45.
11. Girardi G, Berman J, Redecha P, et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. The Journal of clinical investigation 2003;112:1644-54.
12. Girardi G, Yarilin D, Thurman JM, Holers VM, Salmon JE. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. The Journal of experimental medicine 2006;203:2165-75.
13. Derzsy Z, Prohaszka Z, Rigo J, Jr., Fust G, Molvarec A. Activation of the complement system in normal pregnancy and preeclampsia. Molecular immunology 2010;47:1500-6.
14. Haeger M, Unander M, Bengtsson A. Complement activation in relation to development of preeclampsia. Obstetrics and gynecology 1991;78:46-9.
15. Elimian A, Figueroa R, Canterino J, Verma U, Aguero-Rosenfeld M, Tejani N. Amniotic fluid complement C3 as a marker of intra-amniotic infection. Obstetrics and gynecology 1998;92:72-6.
16. Lynch AM, Gibbs RS, Murphy JR, et al. Complement activation fragment Bb in early pregnancy and spontaneous preterm birth. American journal of obstetrics and gynecology 2008;199:354.e1-8.
17. Romero R, Espinoza J, Kusanovic JP, et al. The preterm parturition syndrome.

BJOG : an international journal of obstetrics and gynaecology 2006;113 Suppl 3:17-42.

18. Romero R, Mazor M, Munoz H, Gomez R, Galasso M, Sherer DM. The preterm labor syndrome. Annals of the New York Academy of Sciences 1994;734:414-29.

19. Soto E, Romero R, Richani K, et al. Anaphylatoxins in preterm and term labor. Journal of perinatal medicine 2005;33:306-13.

20. Vaisbuch E, Romero R, Erez O, et al. Activation of the alternative pathway of complement is a feature of pre-term parturition but not of spontaneous labor at term. American journal of reproductive immunology (New York, NY : 1989) 2010;63:318-30.

21. Vaisbuch E, Romero R, Erez O, et al. Fragment Bb in amniotic fluid: evidence for complement activation by the alternative pathway in women with intra-amniotic infection/inflammation. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet 2009;22:905-16.

22. Conde-Agudelo A, Romero R, Nicolaides K, et al. Vaginal progesterone vs. cervical cerclage for the prevention of preterm birth in women with a sonographic short cervix, previous preterm birth, and singleton gestation: a systematic review and indirect comparison metaanalysis. American journal of obstetrics and gynecology 2013;208:42.e1-.e18.

23. Hassan SS, Romero R, Vidyadhari D, et al. Vaginal progesterone reduces the rate of preterm birth in women with a sonographic short cervix: a multicenter, randomized, double-blind, placebo-controlled trial. Ultrasound in obstetrics &

gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology 2011;38:18-31.

24. Romero R. Vaginal progesterone to reduce the rate of preterm birth and neonatal morbidity: a solution at last. Women's health (London, England) 2011;7:501-4.

25. Romero R, Nicolaides K, Conde-Agudelo A, et al. Vaginal progesterone in women with an asymptomatic sonographic short cervix in the midtrimester decreases preterm delivery and neonatal morbidity: a systematic review and metaanalysis of individual patient data. American journal of obstetrics and gynecology 2012;206:124.e1-19.

26. Romero R, Yeo L, Chaemsaithong P, Chaiworapongsa T, Hassan SS. Progesterone to prevent spontaneous preterm birth. Seminars in fetal & neonatal medicine 2014;19:15-26.

27. Romero R, Yeo L, Miranda J, Hassan SS, Conde-Agudelo A, Chaiworapongsa T. A blueprint for the prevention of preterm birth: vaginal progesterone in women with a short cervix. Journal of perinatal medicine 2013;41:27-44.

28. Fonseca EB, Celik E, Parra M, Singh M, Nicolaides KH. Progesterone and the risk of preterm birth among women with a short cervix. The New England journal of medicine 2007;357:462-9.

29. Iams JD. Identification of candidates for progesterone: why, who, how, and when? Obstetrics and gynecology 2014;123:1317-26.

30. Iams JD. Clinical practice. Prevention of preterm parturition. The New England journal of medicine 2014;370:254-61.

31. Meis PJ, Klebanoff M, Thom E, et al. Prevention of recurrent preterm delivery by 17 alpha-hydroxyprogesterone caproate. The New England journal of medicine

2003;348:2379-85.

32. Elovitz MA, Gonzalez J. Medroxyprogesterone acetate modulates the immune response in the uterus, cervix and placenta in a mouse model of preterm birth. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet 2008;21:223-30.

33. Hardy DB, Janowski BA, Corey DR, Mendelson CR. Progesterone receptor plays a major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-kappaB activation of cyclooxygenase 2 expression. Molecular endocrinology (Baltimore, Md) 2006;20:2724-33.

34. Lydon JP, DeMayo FJ, Funk CR, et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes & development 1995;9:2266-78.

35. Thaxton JE, Romero R, Sharma S. TLR9 activation coupled to IL-10 deficiency induces adverse pregnancy outcomes. Journal of immunology (Baltimore, Md : 1950) 2009;183:1144-54.

36. Dudley DJ, Branch DW, Edwin SS, Mitchell MD. Induction of preterm birth in mice by RU486. Biology of reproduction 1996;55:992-5.

37. Tveita AA, Rekvig OP, Zykova SN. Increased glomerular matrix metalloproteinase activity in murine lupus nephritis. Kidney international 2008;74:1150-8.

38. Ludmir J, Sehdev HM. Anatomy and physiology of the uterine cervix. Clinical obstetrics and gynecology 2000;43:433-9.

39. Bigg HF, Rowan AD, Barker MD, Cawston TE. Activity of matrix

metalloproteinase-9 against native collagen types I and III. The FEBS journal 2007;274:1246-55.

40. Czermak BJ, Sarma V, Bless NM, Schmal H, Friedl HP, Ward PA. In vitro and in vivo dependency of chemokine generation on C5a and TNF-alpha. Journal of immunology (Baltimore, Md : 1950) 1999;162:2321-5.

41. Laudes IJ, Chu JC, Huber-Lang M, et al. Expression and function of C5a receptor in mouse microvascular endothelial cells. Journal of immunology (Baltimore, Md : 1950) 2002;169:5962-70.

42. Imada K, Ito A, Sato T, Namiki M, Nagase H, Mori Y. Hormonal regulation of matrix metalloproteinase 9/gelatinase B gene expression in rabbit uterine cervical fibroblasts. Biology of reproduction 1997;56:575-80.

43. Cohen D, Buurma A, Goemaere NN, et al. Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. The Journal of pathology 2011;225:502-11.

44. Lynch AM, Gibbs RS, Murphy JR, Giclas PC, Salmon JE, Holers VM. Early elevations of the complement activation fragment C3a and adverse pregnancy outcomes. Obstetrics and gynecology 2011;117:75-83.

45. Denny KJ, Woodruff TM, Taylor SM, Callaway LK. Complement in pregnancy: a delicate balance. American journal of reproductive immunology (New York, NY : 1989) 2013;69:3-11.

46. Soto E, Romero R, Richani K, et al. Evidence for complement activation in the amniotic fluid of women with spontaneous preterm labor and intra-amniotic infection. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and

Oceania Perinatal Societies, the International Society of Perinatal Obstet 2009;22:983-92.

47. Sato T, Ito A, Mori Y, Yamashita K, Hayakawa T, Nagase H. Hormonal regulation of collagenolysis in uterine cervical fibroblasts. Modulation of synthesis of procollagenase, prostromelysin and tissue inhibitor of metalloproteinases (TIMP) by progesterone and oestradiol-17 beta. The Biochemical journal 1991;275 (Pt 3):645-50.

48. Markiewski MM, DeAngelis RA, Benencia F, et al. Modulation of the antitumor immune response by complement. Nature immunology 2008;9:1225-35.

49. Hughes GC, Martin D, Zhang K, et al. Decrease in glomerulonephritis and Th1-associated autoantibody production after progesterone treatment in NZB/NZW mice. Arthritis and rheumatism 2009;60:1775-84.

50. Guo RF, Riedemann NC, Bernacki KD, et al. Neutrophil C5a receptor and the outcome in a rat model of sepsis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2003;17:1889-91.

51. Guo RF, Riedemann NC, Sun L, et al. Divergent signaling pathways in phagocytic cells during sepsis. Journal of immunology (Baltimore, Md : 1950) 2006;177:1306-13.

52. Ishrat T, Sayeed I, Atif F, Hua F, Stein DG. Progesterone and allopregnanolone attenuate blood-brain barrier dysfunction following permanent focal ischemia by regulating the expression of matrix metalloproteinases. Experimental neurology 2010;226:183-90.

53. McMaster MT, Teng CT, Dey SK, Andrews GK. Lactoferrin in the mouse uterus: analyses of the preimplantation period and regulation by ovarian steroids.

Molecular endocrinology (Baltimore, Md) 1992;6:101-11.

54. Lee YL, Cheong AW, Chow WN, Lee KF, Yeung WS. Regulation of complement-3 protein expression in human and mouse oviducts. Molecular reproduction and development 2009;76:301-8.

55. Novakova P, Zeman M. Progesterone inhibits embryotoxic effect of the complement system. Neuro endocrinology letters 1999;20:105-8.

56. Mesiano S, Welsh TN. Steroid hormone control of myometrial contractility and parturition. Seminars in cell & developmental biology 2007;18:321-31.

57. Ruddock NK, Shi SQ, Jain S, et al. Progesterone, but not 17-alpha-hydroxyprogesterone caproate, inhibits human myometrial contractions. American journal of obstetrics and gynecology 2008;199:391.e1-7.

58. Minorics R, Gaspar R, Gal A, Klukovits A, Falkay G. Progesterone decreases the relaxing effect of the beta3-adrenergic receptor agonist BRL 37344 in the pregnant rat myometrium. Reproduction (Cambridge, England) 2009;138:383-90.

59. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet 2008;371:75-84.

60. Iams JD, Romero R, Culhane JF, Goldenberg RL. Primary, secondary, and tertiary interventions to reduce the morbidity and mortality of preterm birth. Lancet 2008;371:164-75.

61. Timmons B, Akins M, Mahendroo M. Cervical remodeling during pregnancy and parturition. Trends in endocrinology and metabolism: TEM 2010;21:353-61.

62. Gonzalez JM, Franzke CW, Yang F, Romero R, Girardi G. Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. The American journal of pathology 2011;179:838-49.

63. Fichorova RN, Rheinwald JG, Anderson DJ. Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biology of reproduction 1997;57:847-55.

64. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. Endocrine reviews 2000;21:514-50.

65. Choi SJ, Jung KL, Oh SY, Kim JH, Roh CR. Cervicovaginal matrix metalloproteinase-9 and cervical ripening in human term parturition. European journal of obstetrics, gynecology, and reproductive biology 2009;142:43-7.

66. Gonzalez JM, Xu H, Chai J, Ofori E, Elovitz MA. Preterm and term cervical ripening in CD1 Mice (Mus musculus): similar or divergent molecular mechanisms? Biology of reproduction 2009;81:1226-32.

67. Holt R, Timmons BC, Akgul Y, Akins ML, Mahendroo M. The molecular mechanisms of cervical ripening differ between term and preterm birth. Endocrinology 2011;152:1036-46.

68. Hassan SS, Romero R, Tarca AL, et al. The transcriptome of cervical ripening in human pregnancy before the onset of labor at term: identification of novel molecular functions involved in this process. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet 2009;22:1183-93.

69. Liggins GC. Ripening of the cervix. Seminars in perinatology 1978;2:261-71.

70. Kelly RW. Inflammatory mediators and cervical ripening. Journal of reproductive immunology 2002;57:217-24.

71. Malmstrom E, Sennstrom M, Holmberg A, et al. The importance of fibroblasts in remodelling of the human uterine cervix during pregnancy and parturition. Molecular human reproduction 2007;13:333-41.

72. Dubicke A, Akerud A, Sennstrom M, et al. Different secretion patterns of matrix metalloproteinases and IL-8 and effect of corticotropin-releasing hormone in preterm and term cervical fibroblasts. Molecular human reproduction 2008;14:641-7.

73. Gomez-Lopez N, Guilbert LJ, Olson DM. Invasion of the leukocytes into the fetal-maternal interface during pregnancy. Journal of leukocyte biology 2010;88:625-33.

74. Thomson AJ, Telfer JF, Young A, et al. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. Human reproduction (Oxford, England) 1999;14:229-36.

75. Rodriguez HA, Kass L, Varayoud J, et al. Collagen remodelling in the guinea-pig uterine cervix at term is associated with a decrease in progesterone receptor expression. Molecular human reproduction 2003;9:807-13.

76. Becher N, Adams Waldorf K, Hein M, Uldbjerg N. The cervical mucus plug: structured review of the literature. Acta obstetricia et gynecologica Scandinavica 2009;88:502-13.

ABSTRACT

CERVICAL REMODELING/RIPENING AT TERM AND PRETERM DELIVERY: THE SAME MECHANISM INITIATED BY DIFFERENT MEDIATORS AND DIFFERENT EFFECTOR CELLS

by

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Premature cervical remodeling/ripening is believed to contribute to preterm delivery (PTD), the leading cause of perinatal morbidity and mortality. Despite considerable research, the causes of term and PTD remain unclear, and there is no effective treatment for PTD. We tested the hypothesis that complement activation plays a role in cervical remodeling and PTD. We studied cervical remodeling at term.

We studied two mouse models of inflammation-induced PTD. The first model was induced by vaginal administration of lipopolysaccharide (LPS)and the second one by administration of progesterone antagonist RU486. Increased cervical C3 deposition and macrophage infiltration and increased serum C3adesArg and C5adesArg levels were observed in both models when compared to gestational age matched controls. A significant increase in collagen degradation, matrix metalloproteinase 9 (MMP-9) activity and tissue distensibility was observed in the cervix in both models. Mice deficient in complement receptor C5a did not show increased MMP-9 activity and cervical remodeling and did not deliver preterm in response to LPS or RU486, suggesting a role for C5aR in the cervical changes that precede PTD. *In vitro* studies show that

macrophages release MMP-9 in response to C5a. Progesterone diminished the amount of C5aR on the macrophages surface, inhibited the release of MMP-9 and prevented PTD. In addition, macrophage depletion also prevented cervical remodeling and PTD in LPS-treated mice. We found that complement activation is not required for the physiological process that leads to term delivery in mice. Neither increased C3 cervical deposition nor increased C3a and C5a serum levels were observed at term. In addition, macrophages infiltration was found in PTD in contrast to term delivery were no leukocytes were found. Despite the different role of complement and different cellular effector cells, PTD and term delivery share a common dowsntream pathway characterized by increased metalloproteinases (MMPs) release and increased collagen degradation. However, different sources of MMPs were identified. Macrophages are the source of MMPs in PTD while cervical fibroblasts and columnar epithelial cells synthesize MMPs at term delivery. A dramatic diminution in serum progesterone levels precedes parturition at term but not in PTD, suggesting that progesterone withdrawal initiates cervical remodeling at term. On the other hand, MMPs release in PTD is triggered by C5a.

Complement inhibition and supplementation with progesterone may be good therapeutic options to prevent this serious pregnancy complication. Preterm and term cervical remodeling occur through the same mechanism but they are initiated by different mediators and effector cells. That complement activation is required for PTD but not for the physiological process that leads to term delivery, suggests that complement is a potential specific biomarker and selective target to prevent PTD and thus avert neonatal mortality and morbidity.

AUTOBIOGRAPHICAL STATEMENT

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Education

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Post-graduate Training

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Selected Peer Reviewed Publications

1. **Gonzalez JM**, Romero R, Girardi G. Comparison of the mechanisms responsible for cervical remodeling in preterm and term labor. J Reprod Immunol. 2013 Mar;97(1):112-9. PMID: 23312455
2. Hou ZC, Sterner KN, Romero R, Than NG, 1. **Gonzalez JM**, Weckle A, Xing J, Benirschke K, Goodman M, Wildman DE. Elephant transcriptome provides insights into the evolution of eutherian placentation. Genome Biol Evol. 2012 Jan;4(5):713-25. PMID: 22546564
3. **Gonzalez JM**, Dong Z, Romero R, Girardi G. Cervical remodeling/ripening at term and preterm delivery: the same mechanism initiated by different mediators and different effector cells. PLoS One. 2011;6(11):e26877. PMID: 22073213
4. Wildman DE, Uddin M, Romero R, **Gonzalez JM**, Than NG, Murphy J, Hou ZC, Fritz J. Spontaneous abortion and preterm labor and delivery in nonhuman primates: evidence from a captive colony of chimpanzees (Pan troglodytes). PLoS One. 2011;6(9):e24509. PMID: 21949724
5. **Gonzalez JM**, Franzke CW, Yang F, Romero R, Girardi G. Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. Am J Pathol. 2011 Aug;179(2):838-49. PMID: 21801872
6. Yeo L, Romero R, Jodicke C, Kim SK, **Gonzalez JM**, Oggè G, Lee W, Kusanovic JP, Vaisbuch E, Hassan S. Simple targeted arterial rendering (STAR) technique: a novel and simple method to visualize the fetal cardiac outflow tracts. Ultrasound Obstet Gynecol. 2011 May;37(5):549-56. PMID: 20878672
7. Hassan SS, Romero R, Tarca AL, Nhan-Chang CL, Mittal P, Vaisbuch E, **Gonzalez JM**, Chaiworapongsa T, Ali-Fehmi R, Dong Z, Than NG, Kim CJ. The molecular basis for sonographic cervical shortening at term: identification of differentially expressed genes and the epithelial-mesenchymal transition as a function of cervical length. Am J Obstet Gynecol. 2010 Nov;203(5):472.e1-472.e14. PMID: 20817141
8. Mazaki-Tovi S, Vaisbuch E, Romero R, Kusanovic JP, Chaiworapongsa T, Kim SK, Ogge G, Yoon BH, Dong Z, **Gonzalez JM**, Gervasi MT, Hassan SS. Hyperresistinemia - a novel feature in systemic infection during human pregnancy. Am J Reprod Immunol. 2010 May;63(5):358-69. PMID: 20178460
9. Oggè G, Romero R, Kusanovic JP, Chaiworapongsa T, Dong Z, Mittal P, Vaisbuch E, Mazaki-Tovi S, **Gonzalez JM**, Yeo L, Hassan SS. Serum and plasma determination of angiogenic and anti-angiogenic factors yield different results: the need for standardization in clinical practice. J Matern Fetal Neonatal Med. 2010 Aug;23(8):820-7. PMID: 20158394
10. Mazaki-Tovi S, Vaisbuch E, Romero R, Kusanovic JP, Chaiworapongsa T, Kim SK, Nhan-Chang CL, Gomez R, Alpay Savasan Z, Madan I, Yoon BH, Yeo L, Mittal P, Ogge G, **Gonzalez JM**, Hassan SS. Maternal and neonatal circulating visfatin concentrations in patients with pre-eclampsia and a small-for-gestational age neonate. J Matern Fetal Neonatal Med. 2010 Oct;23(10):1119-28. PMID: 20121389
11. Mazaki-Tovi S, Vaisbuch E, Romero R, Kusanovic JP, Chaiworapongsa T, Kim SK, Nhan-Chang CL, Gomez R, Yoon BH, Yeo L, Mittal P, Ogge G, **Gonzalez JM**, Hassan SS. Maternal plasma concentration of the pro-inflammatory adipokine pre-B-cell-enhancing factor (PBEF)/visfatin is elevated in pregnant patients with acute pyelonephritis. Am J Reprod Immunol. 2010 Mar 1;63(3):252-62. PMID: 20085562