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Specific inflammatory microenvironments in the zones of the fetal membranes at term delivery

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**Article Condensation:**

During labor specific immunological microenvironments are created in the zones of the fetal membranes that may be involved in their rupture at the end of gestation.

**Short title:**

Specific microenvironments in fetal membrane zones
Abstract

**OBJECTIVE:** The purpose of this study was to examine the histological and immunological differences between fetal membrane (FM) zones following membrane rupture at term delivery.

**STUDY DESIGN:** FM explants from post-rupture zones (periplacental-PZ, middle-MZ, rupture-RZ) were obtained from women following spontaneous vaginal delivery at term (n=5). Tissues for histology, protein extracts, and RNA were isolated.

**RESULTS:** The collagen distribution decreased and the leukocyte density increased from the PZ to the RZ. T cells were mainly present in the RZ and granulocytes in the MZ. CXCL10, CXCR1, ICAM-1, -2, PSEL, TNF-α and MMP-9 levels were higher in the MZ than in the RZ and PZ (p<0.021). IL-1β and CXCL8 levels were higher in the RZ than in the MZ and PZ (p=0.018 and <0.0001).

**CONCLUSION:** During labor specific immunological microenvironments are created in the zones of the FM that may be involved in their rupture at the end of gestation.

**Keywords:** Parturition, fetal membrane zones, inflammation
Introduction

Normal labor begins at term with intact fetal membranes (FM) which spontaneously rupture near the end of the first stage if there is no intervention. The physiological mechanisms that lead to FM rupture prior to birth are unknown although convention suggests the process is precipitated by the stress of the uterine contractions during labor. However, this fails to explain the 10% of term deliveries and 40% of preterm deliveries where membrane rupture occurs before any uterine contractions begin.

Several studies have indicated that the FM undergo a genetically programmed, biochemically mediated, maturation process near term that is characterized by collagen remodeling and apoptosis. Certain changes are limited to the rupture region of the FM overlying the cervix (termed rupture zone (RZ)). Moore et al. demonstrated that the RZ is a region of physical weakness overlying the cervical opening of the uterus and is characterized by specific markers of increased collagen remodeling and apoptosis. By definition, therefore, the rupture zone should have different characteristics than the intact regions of the FM. It is probable that these regional characteristics develop prior to the onset of the contractions of labor and persist until delivery when the rupture process occurs.

In addition to the morphologic and physiologic changes of labor, an inflammatory microenvironment is created within the FM during parturition. This involves the infiltration of specific leukocyte subsets and the secretion of autocrine and paracrine primary mediators, such as pro-inflammatory cytokines (interleukin 1beta (IL-1β) and tumor necrosis factor alpha (TNF-α)) and chemokines (CXCL8, CXCL10). Mediators that are downstream from the immunological include the prostaglandins and matrix metalloproteinase (MMPs), mainly MMP-9. The expression and activity of MMP-9 selectively increases during labor suggesting it has a role in the physiological and pathological rupture of the FM. In addition, there is increased expression of cell adhesion molecules (CAMs) that associated
with “cellular spreading and homing” \textsuperscript{18,19}. These mediators may be produced and expressed by the resident cells of the reproductive tissues and/or by infiltrating leukocytes. Previously, it was demonstrated that all tissues (amnion and choriodecidua) and zones of the FM and maternal decidua contribute to the creation of an inflammatory microenvironment \textsuperscript{14,20,21}. The aim of this work was to identify the unique characteristics of the microenvironments in the zones of the FM, and to describe them in terms of histology, leukocyte density, and the expression of pro-inflammatory mediators.
Materials and Methods

Subjects and tissue collection

This study was approved by the institutional review board of the Instituto Nacional de Perinatología Isidro Espinosa de los Reyes in Mexico City (Register 212250-02181). Written, informed consent was obtained from each subject prior to inclusion in the study. Subjects were excluded from the study if there was microbiological or clinical evidence of cervico-vaginal or intrauterine infection.

FM that ruptured spontaneously during labor (existence of labor: cervical dilatation $\geq 4$ cm; contractility of the myometrium $\geq 3$ contractions of 40s within a 10 minute (min) period by tocodynamometer) were obtained immediately after normal term ($\geq 37$ weeks) vaginal delivery from uncomplicated pregnancies. The duration of labor was similar (8–15h). To delimit the rupture site we had the help of an obstetrician-gynecologist, who used a vaginal mirror to observe the cervical characteristics. If cervical dilatation was present, she stained the fetal membranes appearing in the middle of the cervical os with a gauze ball soaked in Gentian violet (Sigma-Aldrich, USA). This staining served as a landmark for the true rupture and was confirmed by examination of the fetal membranes after delivery. Tissues were processed within 30 minutes of delivery. In the laboratory, we used two long strips of the FM extending from the RZ to the placental edge and a specimen of the placental chorionic plate for our studies. Each fetal membrane zone was identified according to Figure 1. Multiple explants of 1 cm$^2$ were obtained from the RZ; the middle zone (MZ), halfway between the RZ and the placental edge at least 10-12 cm from the RZ; and from the peri-placental zone (PZ) comprising the 1-2 cm of thickened FM next to the edge of the placenta. FM had well defined rupture sites and did not exhibit abnormalities, separation, or infection. Infection was indicated by the presence of massive polymorphonuclear infiltration and positive microbiological culture. Microbiological tests were performed in tissues by rolling a Dacron
swab on the surface of the membranes. The swabs were cultured onto blood agar plates under aerobic and anaerobic conditions to ensure that tissues were free from infection. Women included in this study had internal monitoring and they were similar in ethnicity (Mexican Mestizo) and parity (primiparous). None of these women received antibiotics for prolonged rupture of fetal membranes, oxytocin augmentation, or immunosuppressive or modulating medications. Thirty five samples were collected, however only five of them were included in this study according to previously described criteria by Malak and Bell. Thirty samples were excluded because the site marked with Gentian violet did not correspond to the rupture site, suggesting that rupture did not occur spontaneously during labor. Rather, the deliveries were resolved via cesarean section or they underwent manual amniorrhexis.

The total number of explants varied between women (15–30) and depended on the length of the RZ and the distance between this zone and the placental edge. Explants were washed carefully and immediately placed in sterile saline solution to eliminate visible blood clots.

**Collagen histochemistry**

FM explants from each zone were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24 h. Tissues were dehydrated and processed for paraffin embedding. Sections (5 μM) were mounted on silane adhesive coated glass slides (Becton Dickinson, USA), dried overnight at room temperature, and stained with picro-sirius red (0.5 g Sirius red F3B [C.I. 35782] in 500 mL saturated aqueous solution of picric acid; Sigma-Aldrich) for 1 h. After washing in 2 changes of acidified water, slides were shaken vigorously by hand. After most of the water had been removed, sections were dehydrated in 3 changes of 100% ethanol, cleared in xylene (Sigma-Aldrich), and mounted in Entellan (Electron Microscopy Sciences, USA). Stained tissue sections were encoded and the blinded analysis was performed under a light microscope connected to an image capture system. Multiple fields were selected from each section.
**Immunofluorescence histochemistry**

Sections from each zone were blocked with PBA (PBS, 1mg mL⁻¹ BSA, 10 mM NaNO₃) for 30 min prior to incubation with conjugated monoclonal antibodies (1:20) for 1 h at 37°C. We determined the phenotype of infiltrated leukocytes using anti-CD45-FITC (clone J33), anti-CD3-PC5 (clone UCHT1), anti-CD14-ECD (clone RM052), anti-CD19-PC7 (clone J4.119), and anti-CD56-PE (clone N901) (10test; Beckman Coulter, USA). We determined the association between MMP-9 (labor mediator) and infiltrated leukocytes in these tissues by double-labeling with anti-MMP-9-FITC (clone 56129; R&D Systems, USA) and anti-CD45-PE (clone J.33; 10test; Beckman Coulter). Sections were washed in 1X PBS (10 mM Na₃PO₄, 150 mM NaCl, pH 7.2; Bio-Rad Laboratories, USA) containing 0.2% Triton (Sigma-Aldrich) (3 buffer changes, 5 min each) and mounted with Vectashield mounting medium (Vector Laboratories, UK) for visualization using the LSM 510 MetaLaser Confocal microscope (Carl Zeiss, UK). The histomorphometric analysis was made using encoded sections.

**FM protein extracts**

Immediately after delivery, FM explants from each zone were washed and special care was taken to maintain the integrity of the FM and not to separate amnion from choriodecidua; both were to be used in all experiments. Each FM explant (1 cm²) was cultured in 1 mL of DMEM, 1% MEM sodium pyruvate, and 1% antibiotic–antimycotic (100 U penicillin, 100 μg streptomycin, 0.25 μg amphotericin B/ml) for 24 h at 37°C in a humid atmosphere containing 5% CO₂. FCS-free conditions were used in all experiments. Following incubation, all explants were homogenized in their culture media using a Polytron (Brinkmann, USA) and pooled. FM extracts from each zone were centrifuged at 14,000 x g, filtered through a 0.2 μm membrane (Corning, USA), and preserved at −70°C until use.

**Chemokine and cytokine concentration**
Concentrations of CXCL8, CXCL10, IL-1β, and TNF-α were measured in FM extracts from each zone using a quantitative microbead assay (Bioplex System Human Cytokine Assay; Bio-Rad Laboratories, USA); broad range standards (1.95–32,000 pg/mL) were used. The protein content of the FM extracts was quantified by the Bradford method, and it was used to normalize the chemokine and cytokine concentrations.

**Total RNA isolation and cDNA synthesis**

FM explants from each zone were placed in RNAlater (Ambion, Austin, TX, USA) in order to preserve RNA, and stored at –70°C until further processing. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Total RNA was quantified by spectrophotometry and RNA integrity was verified by non-denaturing agarose gel electrophoresis. cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) using random hexamer primers. The reverse transcription reaction was carried out (25°C, 10 min; 55°C, 30 min; 85°C, 5 min) using the Mastercycler Gradient equipment (Eppendorf, Hamburg, Germany). cDNA was stored at –20°C until use.

**Real-time PCR**

Quantitative real-time PCR was performed using the Light Cycler 480, the Probes Master kit, and TaqMan Probes (hydrolysis probes labeled with fluorescein) according to the manufacturer’s protocol (Roche Applied Science). Specific primers for mRNA sequences of different genes were designed with the ProbeFinder software accessible at www.universalprobelibrary.com. TaqMan probes were used (Table 1). ACTB gene was the reference for normalizing purposes. To avoid false positive signals from possible residual genomic DNA, all primers were designed to have intron spanning sequences. Five hundred nanograms of sample cDNA were added to each reaction. Real-time PCR conditions were as follows: 1 cycle at 95°C, 10 min; 55 cycles of denaturation (95°C, 10 s); annealing (60°C, 30
s); and extension (72°C, 1 s). Relative quantification of each molecule was calculated with the Light Cycler 480 SW 1.5 software (Roche Applied Science).

**Statistical Analysis**

The data were examined initially by the Shapiro-Wilk test for normal distribution. Kruskal Wallis Test was used when data were not normally distributed. ANOVA and post-hoc test were used when data were normally distributed. Statistical analysis was performed using SPSS, version 17.0. A P value of ≤ 0.05 was considered statistically significant.
Results

FM structure, collagen distribution, and leukocyte density

Distinctive zonal histological changes are evident in Figure 1. The PZ conserved the classic FM structure and collagen distribution in comparison to the MZ and RZ. In the PZ the leukocyte density in the choriodecidua was minimal. In the MZ, the chorion was considerably thinner than in the PZ, although the amnion’s spongy layer was conserved. There was an apparent increase in leukocyte density in the choriodecidua of the MZ when compared to the PZ. In the RZ there was considerable loss of tissue integrity which was evident in the spongy layer and also evident by the decreased collagen content. The histological findings in this zone correlated with an increase in leukocyte density in the choriodecidua. Overall, the leukocyte density increased from the placenta to the RZ.

Phenotype of infiltrated leukocytes

Granulocytes (CD45^+CD3^-CD14^-CD19^-CD56^-) were present in all zones, but mainly in the MZ. Monocytes/macrophages (CD45^+CD14^+) were found sporadically in the PZ. Interestingly, T cells (CD45^+CD3^+) were present in the MZ and RZ, and their density was apparently increased in the RZ. B cells (CD45^-CD19^+) and NK cells (CD45^-CD56^-) were not observed in the FM zones (Figure 2).

Chemokine secretion and expression

CXCL8 secretion was higher in the RZ compared to the MZ and PZ (p < 0.0001). CXCL10 secretion was also greater in the MZ and RZ than in the PZ, however this increase was not significant (Figure 3).

The mRNA expression of CXCL8, CXCL10 and their receptors showed significant differences between zones. There were higher levels of both chemokines (CXCL8 and CXCL10) and their receptors (CXCR1; CXCR2 and CXCR3, respectively) in the MZ.
compared to the PZ and RZ. However, this difference was only significant for CXCL10 and CXCR1 \((p = 0.027\); Figure 4a-e).

**CAMs expression**

There were significant differences in the expression of three CAMs between the FM zones. Levels were significantly higher in the MZ for ICAM-1 \((p = 0.039)\), ICAM-2 \((p = 0.027)\), and PSEL \((p = 0.027)\) than in the RZ and PZ (Figure 4f-h). ICAM-3, VCAM, ESEL, LFA-1, and Mac-1 levels were not significantly different between groups (data not shown).

**Expression of labor mediators**

TNF-\(\alpha\) mRNA abundance was higher in the MZ than in the RZ and PZ \((p = 0.021)\) (Figure 5A). However, TNF-\(\alpha\) secretion was not different between zones (Figure 5B).

IL-1\(\beta\) mRNA abundance was greater in the RZ than in the MZ and PZ \((p = 0.018)\) (Figure 5A). IL-1\(\beta\) secretion also appeared to be higher in the RZ than in the MZ and PZ (Figure 5B). MMP-9 secretion appeared to be more abundant in the RZ than in the MZ and PZ (Figure 6A); however, the mRNA levels of this protein were higher in the MZ than in the RZ and PZ \((p < 0.027)\); Figure 6B). Interestingly, there was an association between MMP-9 protein levels and leukocyte density which also increased from the PZ to the RZ (Figure 6A).
Comment

Accumulating evidence suggests that human parturition represents an inflammatory response which is characterized by the presence of infiltrated leukocytes and pro-inflammatory mediators in the gestational tissues\textsuperscript{10, 12, 13, 23}. These gestational tissues include the FM which retain amniotic fluid, secrete substances both into the amniotic fluid and toward the uterus, and guard the fetus against infection ascending the reproductive tract\textsuperscript{2}. Attached to the chorion is the decidua or choriodecidua, which contains leukocytes. Our preliminary data shows that the choriodecidual leukocyte density increases gradually at the end of gestation, mostly during labor\textsuperscript{14, 24}. The choriodecidual leukocytes mostly have a CD3\textsuperscript{+} phenotype, which suggests that this leukocyte subset participates in the creation of a choriodecidual microenvironment at the time of delivery\textsuperscript{10, 24}; however, the components of this microenvironment are still unclear. Previous reports demonstrated that human FM contain a biomechanically weak zone overlying the cervix in which there is increased collagen remodeling and apoptosis relative to other membrane areas\textsuperscript{7, 9, 25}. In this study, we described the histology, leukocyte density, and expression of pro-inflammatory mediators in different zones of the FM collected after normal term delivery. We found that each zone of the FM has a different microenvironment. The PZ conserved its collagen distribution, while the leukocyte density and expression of pro-inflammatory mediators was minimal in comparison with the other zones. The MZ lost its collagen distribution, however it conserved the spongy layer (which is between the amnion and the chorion), and therefore its integrity. We observed higher levels of expression of pro-inflammatory markers in the MZ than in other zones and we found leukocyte density (mainly granulocytes and some T cells) was greater in the MZ than in the PZ but not the RZ. In the RZ we observed that collagen integrity was completely lost (the spongy zone was dissociated) which correlated with high levels of IL-1\textbeta, CXCL8 and a massive infiltration of leukocytes, mostly T cells.
Our data indicate that the PZ does not undergo large changes in collagen distribution. This finding is in agreement with previous studies which also found minimal and infrequent damage in this zone. We observed the macrophages sporadically distributed in this zone. Several studies have demonstrated the infiltration of macrophages into the FM in late gestation; however they did not consider the possibility of different quality rupture zones. Monocytes/macrophages have multiple functions including the secretion of mediators such as IL-1, IL-6, and CXCL8. Although leukocyte density was minimal in the PZ, it was associated with the expression of MMP-9. Even though MMP-9, TNF-α, and IL-1β levels in the PZ were minimal, their occurrence may be indicative of physiological mechanisms that contribute to the final rupture of these tissues during labor.

We found that the MZ expressed many pro-inflammatory mediators such as CXCL10, CXCR1, TNF-α, and MMP-9 with significant abundance. It has previously been demonstrated that such mediators participate in the recruitment of leukocytes to the FM and to the degradation of these tissues. Since expression normally precedes secretion, we suggest that the MZ is a step back from the RZ, where specific mediators are actively expressed that weaken the FM. Our data indicate that granulocytes (and some T cells) infiltrate the MZ. This is in accordance with previous reports, however this is the first time that granulocyte infiltration has been localized to the MZ of the FM. It is known that granulocytes, specifically neutrophils, represent a rich source of inflammatory mediators during labor, including plasminogen activators, eicosanoids, collagenase, elastase, and pro-inflammatory cytokines such as IL-1β and TNF-α. It is also known that granulocytes are present throughout pregnancy and that their density increases during late gestation. Furthermore, neutrophils are thought to play an important role in collagenolysis during human cervical ripening. Our data show that granulocyte infiltration into the FM is strongly associated with the expression of labor mediators such as TNF-α and MMP-9. This
correlates with previous reports which indicate that neutrophils are a rich source of extracellular matrix proteases including MMP-9. This enzyme has been associated with the physiological and pathological rupture of the FM \textsuperscript{16, 17}. These labor mediators (TNF-\textit{\alpha} and MMP-9) participate in placent al abruption and are associated with preterm premature membrane rupture \textsuperscript{40-42}. Several studies have associated neutrophil influx in humans with CXCL8 levels \textsuperscript{43, 44}. Our data also show that this chemokine is mainly secreted in the RZ; however CXCL8 receptors (mainly \textit{CXCR1}) are over-expressed in the MZ, which may mean granulocyte recruitment starts in this zone and extends to the RZ. In addition, we observed that the CAMs: \textit{ICAM-1}, \textit{ICAM-2}, and \textit{PSEL}, are highly expressed in the MZ. It is known that granulocyte recruitment into reproductive tissues is associated with \textit{ICAM-1} expression \textsuperscript{19}. Taken together, these data suggest that the MZ participates actively in the creation of a choriodecidual microenvironment that promotes the rupture of the FM during labor.

In the RZ, we found that the collagen structure is completely lost, that this zone is enriched with infiltrated T cells (and some granulocytes), and it contains high levels of IL1\textbeta, CXCL8, and MMP-9. Our data prompts us to suggest that in the cervical zone there are regulatory processes involving immunological mediators. During term pregnancy, 45–50\% of the leukocytes in the decidua basalis are CD3\textsuperscript{+}. This is one of the most abundant leukocyte populations \textsuperscript{45-47} and is the result of the pregnancy-induced expansion of the total number of regulatory T cells \textsuperscript{48, 49}. These cells are thought to protect the fetus from maternal immune attack at the maternal-fetal interface since they express markers of activation, and the regulatory T-cell subpopulation, CD25\textsuperscript{}CD4\textsuperscript{+}. CD25\textsuperscript{+}CD4\textsuperscript{+} cells are a specialized T cell population that not only suppress auto aggressive immune responses but may prevent graft rejection due to transplantation antigen intolerance \textsuperscript{47, 50, 51}. Recruitment of T cells may depend on several chemoattractants including CCL5 \textsuperscript{52}, IL-16 \textsuperscript{53}, CXCL9, CXCL10, and CXCL11 \textsuperscript{54, 55}. We recently demonstrated that CXCL10 and CCL5 levels increase in
intrauterine tissues during human labor at term compared to those in the absence of labor. This suggests that these chemokines, particularly CXCL10, may be the main chemotactic homing signals for T cell recruitment during labor. Although our data did not show significant differences in CXCL10 expression levels between zones, our data do show that the RZ contains high protein levels of CXCL8. It is known that oligomerization of chemokines is essential for the aspect of chemokine function that is independent of direct receptor binding, in vivo; therefore, we suggest that CXCL8 may potentiate the action of CXCL10. Thus, T cell recruitment may depend on the interaction of CXCL8-CXCL10 and, perhaps, with other chemokines that were not determined here. In the RZ, we observed higher expression and secretion levels of IL-1β than in other zones. This is in accordance with previous studies which suggest that IL-1β, together with TNF-α, induce collagen remodeling and apoptosis in the RZ. Our data also showed that the massive leukocyte T cell infiltration is associated with MMP-9. Both these and our preliminary data show for first time in the FM that MMP-9 is associated with leukocyte infiltration and the secretion of pro-inflammatory cytokines such as IL1β in the choriodecidua of the RZ. As mentioned, this enzyme is involved in the collagenolysis of these tissues. Furthermore, MMP-9 has been associated with the regulation of IL1β concentrations, since it degrades the active form of IL1β to its inactive form. These data strengthen our hypothesis that there is a specific regulatory microenvironment in the RZ where the T cells secrete regulatory cytokines such as IL1β. These events may play a role in the regulation of MMP-9 secretion and activity.

In summary, we have shown that during labor a clear gradient of leukocytes, chemokines, CAMs, and labor mediators exists in the FM extending from the placenta to the rupture site. Leukocytes and resident cells contribute to the creation of specific microenvironments in each zone that lead to the rupture of these tissues and the termination of gestation. In addition, we documented a massive T-cell infiltration into the rupture site of the fetal
membranes. These findings allow us propose that T cells, granulocytes, and resident cells contribute to the creation of specific inflammatory microenvironments in the zones of the fetal membranes that may promote FM rupture at the end of gestation.
References


**Figure Legends**

Figure 1. Histological differences between the fetal membrane zones (PZ, MZ, and RZ). Collagen distribution decreased from the PZ to the RZ, ordered tissue structure was lost, and the leukocyte density increased from the PZ to RZ. White arrows – collagen distribution; blue arrows – leukocyte density. Light Microscopy: magnification x 100. Data are representative of three or more independent experiments with five human tissues per group.

Figure 2. Phenotype of infiltrating leukocytes in the choriodecidua of the fetal membrane zones (PZ, MZ and RZ). Photomicrographs of the choriodecidua. The structure of the choriodecidua is shown (bright field). Each subpopulation of leukocytes was identified by double labeling: anti-CD45-FITC&anti-CD14-ECD; CD45&anti-CD56-PE; CD45&anti-CD19-PC7; CD45&anti-CD3-PC5. Leukocyte subsets are identified with arrows. There were few monocytes in the PZ. Granulocytes were present in all zones but were mainly observed in the MZ, and T cells were mostly present in the RZ. Bars, 20μm. Confocal Microscopy: magnification x 200. Data are representative of three or more independent experiments with five human tissues per group.

Figure 3. Chemokine concentrations in the fetal membrane zones (PZ-white bar, MZ-gray bar, and RZ-black bar). CXCL8 concentration in the RZ was higher than in the MZ and PZ \( (p < 0.0001) \). Data shown are means ± SEM of determinations in duplicate with five human tissues per group. Significance was determined using the ANOVA test.

Figure 4. Relative expression of chemokines, their receptors and CAMs in the fetal membrane zones (PZ-white bar, MZ-gray bar, and RZ-black bar). Quantification of **CXCL8**, **CXCL10**, **CXCR-1**, -2, -3, **ICAM-1**, -2 and **PSEL** mRNA was performed by real-time PCR.
Relative expression is shown on the y-axis, and each zone is represented on the x-axis. **CXCL10, CXCR1, ICAM-1, -2, and PSEL** levels were greater in the MZ than in the RZ and PZ \( (p = ¥0.027, \pi0.027, \tau0.039) \). Data are expressed as relative expression using as reference the *ACTB* gene. Data shown are medians ± quartiles of determinations in duplicate with five human tissues per group. Significance was determined using the Kruskal-Wallis test.

Figure 5. A) Relative expression of **TNF-α** and **IL1β** in the fetal membrane zones (PZ-white bar, MZ-gray bar, and RZ-black bar). Quantification of **TNF-α** and **IL1β** mRNA was performed by real-time PCR. Relative expression is shown on the y-axis and each zone is represented on the x-axis. **TNF-α** was greater in the MZ than in the RZ and PZ \( (p = ¥0.027) \). **IL1β** was higher in the RZ than in the MZ and PZ \( (p = ¥0.018) \). Data are expressed as relative expression using as reference *ACTB* gene. Data shown are medians ± quartiles. Significance was determined using the Kruskal-Wallis test. B) **TNF-α** and **IL-1β** concentrations in the fetal membrane zones. **IL1β** concentration appeared to be higher in the RZ than in the MZ and PZ. Data shown are means ± SEM. Determinations in duplicate with five human tissues per group.

Figure 6. A) Immunolocalization of **MMP-9** (FITC) in the infiltrating leukocytes in the choriodecidua in the three zones (PZ, MZ, and RZ). Photomicrographs are of the choriodecidua. The structure of the choriodecidua is shown (bright field). **MMP-9** and its association with choriodeci dual total leukocytes (CD45+) demonstrated an apparent increase from PZ to RZ. Bars, 20μm. Confocal Microscopy: magnification x200. B) Relative expression of **MMP-9** in the fetal membrane zones (PZ-white bar, MZ-gray bar, and RZ-black bar). Quantification of **MMP-9** mRNA was performed by real-time PCR.
expression is shown on the y-axis and MMP-9 expression from the three zones is represented on the x-axis. MMP-9 was greater in the MZ than in the RZ and PZ ($p = 0.027$). Data are expressed as relative expression using as reference $ACTB$ gene. Data shown are medians ± quartiles and representative of independent experiments and determinations with five human tissues per group. Significance was determined using the Kruskal-Wallis test.
Figure 2

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Figure 3

The graph shows the levels of CXCL8 and CXCL10 proteins per mg of protein for different groups labeled as PZ, MZ, and RZ. The x-axis represents the two cytokines, CXCL8 and CXCL10, while the y-axis represents the protein concentration in pg/mg. The bars indicate the mean values with error bars representing the standard deviation. The graph visually compares the levels of these cytokines across the different groups.
Table 1. Primers and TaqMan probes used by the quantification of the mRNA abundance of pro-inflamatory mediators in the fetal membranes zones

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<td>Fwd: CAA TGA ATT CCA ACG TCA GC  Rev: ACC AAA GTC GGT TGC AGT GT</td>
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<td><strong>ICAM-3</strong></td>
<td>Fwd: GGT ACC ATC CCG TGT GTG G  Rev: GAA CTC CTG CCC CTG GAC</td>
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<td><strong>VCAM-1</strong></td>
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<td><strong>ESEL</strong></td>
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<td><strong>PSEL</strong></td>
<td>Fwd: CCT TCA GGA TGG ACA GC  Rev: TGT AGT TCT GAG CAT TCC ACA GC</td>
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<td><strong>LFA-1</strong></td>
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<td><strong>IL-1β</strong></td>
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<td><strong>MMP-9</strong></td>
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<td><strong>ACTB</strong></td>
<td>Fwd: ATT GGC AAT GAG CGG TTC  Rev: GGA TGC CAC AGG ACT CCA T</td>
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