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Evidence for a role for the adaptive immune response in human term parturition

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Abstract

PROBLEM—Spontaneous labor at term involves leukocyte recruitment and infiltration into the choriodecidua; yet, characterization of these leukocytes and their immunological mediators is incomplete. The purpose of this study was to characterize the immunophenotype of choriodecidual leukocytes as well as the expression of inflammatory mediators in human spontaneous parturition at term.

METHOD OF STUDY—Choriodecidual leukocytes were analyzed by FACS, immunohistochemistry, and RT-PCR in three different groups: (i) preterm gestation delivered for medical indications without labor; (ii) term pregnancy without labor; and (iii) term pregnancy after spontaneous labor.

RESULTS—Two T-cell subsets of memory-like T cells ($CD3^+CD4^+CD45RO^+$ and $CD3^+CD4^-CD8^-CD45RO^+$ cells) were identified in the choriodecidua of women who had spontaneous labor. Evidence for an extensive immune signaling network composed of chemokines (CXCL8 and CXCL10), chemokine receptors (CXCR1-3), cytokines (IL-1β and TNF-α), cell adhesion molecules, and MMP-9 was identified in these cells during spontaneous labor at term.

CONCLUSIONS—The influx of memory-like T cells in the choriodecidua and the evidence that they are active by producing chemokines and cytokines, and expressing chemokine receptors, cell adhesion molecules, and a matrix-degrading enzyme provides support for the participation of the adaptive immune system in the mechanisms of spontaneous parturition at term.

Keywords

Chemokines; choriodecidua; chorion; cytokines; decidua; labor; leukocytes; memory T cells; pregnancy; T cells

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INTRODUCTION

Human parturition is characterized by an inflammatory response that has been demonstrated in the cervix,[1–11] myometrium,[9,10,12–14] and choriodecidua.[9,10,12,15–22] Indeed, leukocyte infiltration has been demonstrated in all these tissues in humans.[1–22] Moreover, an unbiased analysis of gene expression (transcriptome) of these tissues has also demonstrated that labor at term is associated with an inflammatory signature, as there is enrichment of gene ontology categories associated with inflammation.[23–32] Importantly, neutrophil chemokines and other inflammatory mediators are over-expressed in the chorioamniotic membranes, even in the absence of leukocyte infiltration (histologic chorioamnionitis).[23,24,32] Similarly, an inflammatory signature has been observed in the myometrium of women in early labor without histologic evidence of inflammation of the chorioamniotic membranes.[31] In contrast, in the uterine cervix, degradation of extracellular matrix appears to be the key process for ripening[25,26,29,30] and inflammation is involved in cervical dilatation during labor after ripening has occurred, as well as postpartum repair.[27,33,34]

The choriodecidua is strategically located, as it represents an area of direct contact between maternal (decidua) and fetal tissues (chorion or trophoblast). The areas of contact are (i) the decidua parietalis, which lines the uterine cavity not covered by the placenta, and which is in juxtaposed to the chorion laeve, and (ii) the decidua basalis, which is in the basal plate of the placenta and is invaded by interstitial trophoblast.[35] The intimacy of these areas of contact creates the conditions for fetal antigenic exposure to the maternal immune system.[36–43] Tolerance of the fetal semi-allograft requires modulation of the local immune response for successful reproduction.[39–42,44–57] Rejection of the semi-allograft has been implicated as a mechanism of disease in pregnancy complications, such as recurrent spontaneous abortion, preterm labor, preeclampsia, and fetal death[58–69]; however, the precise mechanisms for both tolerance and maternal anti-fetal rejection are poorly understood.

The decidua is composed of typical stromal-type cells, glandular cells and leukocytes.[70–73] The phenotype of decidual leukocytes during spontaneous labor at term has not been completely characterized, and the emphasis has been on the characterization of the cells of the innate limb of the immune response [neutrophils and macrophages].[74–79] There is a paucity of information about cells of the adaptive immune response (T and B cells) in the decidua during labor. Previous studies conducted by our group and others have demonstrated the importance of the choriodecidual microenvironment in spontaneous parturition in humans, strengthening the role of the innate immune system in labor.[17,19,21,22,80–83]

Leukocyte recruitment appears to be the first step in the conditioning of this microenvironment as term approaches. We have proposed that activated leukocytes extravasate from the local circulation into the choriodecidua in preparation for labor.[10,19,84–86] This is accomplished by selective chemotaxis of maternal peripheral leukocytes,[19,21,22,86] and is mediated by specific chemokine expression, which results in the infiltration of neutrophils and macrophages.[19,23,67,86–89] Once specific leukocyte subsets are recruited into the choriodecidua, they form clusters after expressing selective cell adhesion moelcules (CAMs).[8,18,86,90] labor would result by the secretion of at least two waves of activating and effector molecules. Some of these activating molecules include autocrine and paracrine mediators such as pro-inflammatory cytokines, IL-1β (interleukin-1 beta), TNF-α (tumor necrosis factor-alpha), and chemokines such as CXCL8 (chemokine C-X-C motif ligand 8 or IL8) and CXCL10 (or IP-10).[19,21,67,91–93] On the other hand, prostaglandins and MMPs (matrix metalloproteinases) act as effector molecules.[16,94–102] Together, these molecules elicit local cell responses resulting in the amplification of signaling, the induction

of myometrial contractions and extracellular matrix degradation in the cervix and fetal membranes, which promote spontaneous labor and, eventually, delivery.[103–105]

However, a fundamental question that remains unaddressed is whether the adaptive immune system is involved in physiologic parturition. The issue of whether the onset of labor represent 'rejection' of the semi-allograft has remained a speculation for decades.[68,106,107]

This study was conducted to examine the inflammatory microenvironment in the choriodecidua during spontaneous labor at term with a particular focus on the adaptive immune response. Specifically, we aimed to (i) determine the number of phenotype of the infiltrating leukocytes, (ii) identify key chemokines and receptors, and CAMs participating in the leukocyte recruitment/homing, and (iii) analyze the association of these infiltrating leukocytes with the inflammatory microenvironment found in the choriodecidua during spontaneous labor at term pregnancy.

MATERIALS AND METHODS

PATIENTS AND TISSUES

Fetal membranes (amnion and choriodecidua) were collected during indicated cesarean deliveries from women in the following groups: (i) preterm gestation with indications for preterm delivery (designated as preterm gestation group or PTG, 32.9 ± 2.4 weeks, $n = 5$); (ii) term gestation not in labor (group TNL), undergoing cesarean delivery for obstetrical indications such as a previous cesarean delivery (38.4 ± 1.1 weeks, $n = 7$); and (iii) term gestation who underwent spontaneous labor and delivered vaginally without complications (group TL, 39.6 ± 0.31 weeks, $n = 6$).

Samples were excluded from the study if there was microbiological or clinical evidence of cervicovaginal or intrauterine infection. Inflammation of the chorioamniotic membranes was identified by the presence of a massive polymorphonuclear infiltration and a positive culture for microorganisms. Cultures were performed by rolling a Dacron swab on the surface of the membranes. The swabs were cultured onto blood agar plates under aerobic and anaerobic conditions. Women included in this study belonged to the same ethnic group (Mexican mestizo) and were primiparous. None of these women received oxytocin, antibiotics, or immunosuppressants.

This study was approved by the IRB of the Instituto Nacional de Perinatologia Isidro Espinosa de los Reyes in Mexico City, Mexico. Written informed consistent was obtained from each patient prior to inclusion in the study. The IRB has a Federal Wide Assurance. This study was considered exempt for review by the IRB of Wayne State University.

ISOLATION OF CHORIODECIDUAL LEUKOCYTES

Fetal membranes were washed and immediately placed in sterile saline solution to eliminate blood clots. Choriodecidual leukocyte suspensions were prepared by scraping the choriodecidua using a plastic cell scraper (Corning Incorporated, Life Sciences, Lowell, MA, USA).[72] The material was then suspended in 1 mL of 1x PBS (Bio-Rad Laboratories, Hercules, CA, USA) + 0.5% bovine serum albumin + 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, USA) and filtered with a MACS pre-separation filter (30 μm) (Miltenyi Biotec, Auburn, CA, USA). Choriodecidual leukocyte suspensions were centrifuged at 300 × *g* for 10 min and resuspended in 80 μL of 1 x PBS. Finally, 20 μL of anti-CD45 MAb coupled with MACS magnetic beads (Miltenyi Biotec) were added, mixed, and incubated for 20 min at 4 °C. Choriodecidual leukocytes ($CD45^+$ cells) were purified under MS MACS columns and magnetic cell sorting (Miltenyi Biotec). Viability (90–95%) of leukocytes was assessed with the trypan blue exclusion assay.

QUANTIFICATION OF CHORIODECIDUAL LEUKOCYTES

Prior to isolating the choriodecidual leukocytes, fetal membranes from each group of women were spread and measured according to the details described in Fig. S1A. The area of the fetal membranes was calculated following the description of Fig. S1A. Choriodecidual leukocytes were isolated and counted with an automatic cell counter (AC•T 5diff CP Hematology Analyzer; Beckman Coulter, Brea, CA, USA).

PHENOTYPE OF CHORIODECIDUAL LEUKOCYTES

Purified choriodecidual leukocytes were resuspended in 100 μL of 1 x PBS and stained using conjugated monoclonal antibodies (10 μL each) for 15 min on ice, in the dark. The panel of antibodies used in this study is described in Table S1. Choriodecidual leukocytes were then fixed using 500 μL of OptiLyse B (Beckman Coulter), washed, and resuspended in 500 μL of 1 x PBS to be analyzed by flow cytometry (FC-500, Beckman Coulter). The phenotype of leukocytes was analyzed within the $CD45^+$ and $CD3^+$ region, respectively (Fig. S1B).

IMMUNOHISTOCHEMISTRY

Fetal membranes (amnion and choriodecidua) were cut into ~3 cm^2 and washed gently in 1 x PBS. Tissues were fixed in 10% neutral-buffered formalin for about 24 hr, rinsed and stored in 70% ethanol. Tissues were then processed for paraffin embedding. Sections (5 μm) were mounted on silane adhesive coated glass slides (Becton Dickinson, Franklin Lakes, NJ, USA) and dried at 37 °C for 12 hr. Sections were blocked with 1 x PBS/1 mg/mL bovine serum albumin/10 mM NaN_3 for 30 min prior to incubation with conjugated monoclonal antibodies at recommended concentrations for 1 hr at 37 °C. The phenotype of infiltrated leukocytes was determined using double labeling: CD45-FITC with either CD3-PC5, CD56-PE, CD14-PE-Texas Red or CD19-PC7 (Table S1). Subsets of T cells were localized in these tissues using triple labeling: CD3-FITC, CD4-PC5 and CD8-PE. We also tested whether these cells were naïve- or memory-like T cells using double labeling: CD4-FITC or CD8-PC5 with CD45RA0PE or CD45RO-PE (Table S1).

IL-1β, TNF-α and MMP-9 were also localized using double labeling with monoclonal antibodies at recommended concentrations (Table S1). In addition, we identified the leukocytes that produce MMP-9 using double labeling with the following monoclonal antibodies: MMP-9-FITC and CD45-PE or CD3-PC5 (Table S1).

Sections were finally washed in 1 x PBS containing 0.2% Triton (Sigma-Aldrich) (three buffer changes, 5 min each) and mounted with Vectashield mounting medium (Vector Laboratories, Pet, UK) for visualization using the LSM 510 MetaLaser confocal microscope (Carl Zeis, Herts, UK).

RNA ISOLATION AND cDNA SYNTHESIS

Purified choriodecidual leukocytes were placed in RNAlater (Ambion, Austin, TX, USA) and stored at −70 °C until further processing. Total RNA (ribonucleic acid) was isolated using Trizol reagent (Invitrogen, Grand Island, NY, USA) following the manufacturer's protocol. Total RNA was quantified by spectrophotometry, and RNA integrity was verified by non-denaturing agarose gel electrophoresis. cDNA (complementary deoxyribonucleic acid) was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany), using random hexamer primers. Revers transcription reaction was carried out in Mastercycler Gradient equipment (Eppendorf, Hamburg, Germany) at 25 °C for 10 min, 55 °C for 30 min, and 85 °C for 5 min. cDNA was stored at −20 °C and was used the next day.

REAL-TIME PCR

Quantitative real-time PCR (polymerase chain reaction) was performed in a Light Cycler 2.0 instrument using Light Cycler TaqMan Master kit and TaqMan Probes following the manufacturer's protocol (Roche Applied Science). Specific primers for mRNA (messenger RNA) sequences of different genes were designed using the ProbeFinder software accessible at www.universalprobelibrary.com (Table S2). *ACTB* (beta-actin) was used as a reference gene. All primers were designed to have intron spanning sequences, to avoid false positive signals from possible residual genomic DNA. Five hundred nanograms of sample cDNA were added to each reaction. Real-time PCR conditions were as follows: one cycle at 95 °C for 10 min and 45 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 Software 4 (Roche Applied Science).

STATISTICAL ANALYSIS

A Shapiro-Wilk test was performed to determine whether the data were normally distributed. ANOVA and *post hoc* tests were used when this was the case. Kruskal-Wallis tests were used, followed by Mann-Whitney *U*-tests, when the data were not normally distributed. Statistical analysis was performed using SPSS (IBM Corp, SPSS Inc, Chicago, Il, USA), version 18.0. A *P*-value of ≤ 0.05 was considered statistically significant.

RESULTS

T-CELL PROPORTIONS INCREASE IN THE CHORIODECIDUA DURING SPONTANEOUS LABOR

The leukocyte density was significantly higher in tissues from patients in term gestation than in preterm gestation ($P = 0.03$); however, there was no significant difference in leukocyte density before or after spontaneous labor at term (Fig. 1a). we then investigated whether the leukocyte subset proportions change between the 3 clinical groups. The proportion of T cells and monocytes were lower in tissues from women in preterm gestation than in term gestation not in labor ($P < 0.0001$ and 0.023). In contrast, granulocyte proportions were higher in tissues from women in preterm gestation than in term gestation not in labor ($P < 0.0001$). While the proportion of T cells was higher, the proportion of granulocytes was lower in tissues from women who had undergone spontaneous labor than in those who did not have labor ($P = 0.006$ and 0.03). Therefore, the proportion of T cells in the choriodecidua increase as a function of gestational age (Fig. 1 b).

CHORIODECIDUAL LEUKOCYTES INCLUDE $CD4^+$ T CELLS AT TERM PREGNANCY AND ALSO $CD4^-CD8^-$ T CELLS DURING SPONATENOUS LABOR AT TERM

Next, we investigated the phenotype of T cells in each clinical group. Within T cells, the proportion of $CD4^+$ T cells were higher in tissues from women in term (regardless of whether they had undergone labor) than in preterm gestation ($P < 0.0001$ each). In contrast, the proportion of double-negative $CD3^+CD4^-CD8^-$ (DN) T cells were higher in tissues from patients in preterm gestation than in term gestation ($P < 0.001$ each). Importantly, the proportion of DN T cells were higher in the choriodecidua obtained from patients who had undergone spontaneous labor than in those who had not experienced labor ($P = 0.05$). The proportion of $CD8^+$ T cells did not change significantly among clinical groups (Fig. 1c).

Although we localized T cells in both amniochorion and choriodecidua, they were more abundant in the choriodecidua. In amniochorion, T cells were more abundant in women in term gestation than in preterm gestation. In choriodecidua, T cells were more abundant in women who had undergone spontaneous labor than in women who had not experienced

labor (Fig. 2). Monocytes were sporadically detected at term gestation in very low numbers, and B cells and NK cells were undetectable by confocal microscopy (data not shown).

Next, we localized T-cell subsets in the choriodecidua. $CD4^+$ T cells were higher in tissues from patients in term gestation than in preterm gestation (Fig. 3a), and $CD8^+$ T cells were undetectable or barely detectable (data not shown).

CHORIODECIDUAL T CELLS EXPRESS $CD4RO^+$ 'MEMORY-LIKE T CELLS' DURING BOTH TERM PREGNANCY AND SPONTANEOUS LABOR AT TERM

Choriodecidual T cells, including mostly $CD4^+$ T cells, expressed CD45RO, a memory-like marker. Although there seems to be more $CD4^+$ memory-like T cells in tissues from women in term gestation with spontaneous labor than in those without labor, the increase observed was not consistent between all histologic samples (Fig. 3b). Such findings were also supported by flow cytometry analysis (Fig. 1c).

In addition, we observed a few $CD45RO^+$ cells that did not express CD4 or CD8 in tissues from patients who had undergone spontaneous labor. These cells were considered to represent memory-like DN T cells (data not shown).

CHORIODECIDUAL LEUKOCYTES EXPRESS SPECIFIC CHEMOKINES/RECEPTORS DURING SPONTANEOUS LABOR AT TERM

As most of the infiltrated cells were T cells and granulocytes, we determined the mRNA relative expression of chemokines and receptors and related to neutrophil and T-cell recruitment in isolated choriodecidual leukocytes. mRNA expression of the chemokines *CXCL8* and *CXCL10*, and their receptors *CXCR1* (chemokine C-X-C motif receptor 1), *CXCR2*, and *CXCR3* was determined. For each chemokine and its receptor(s), the expression was higher in choriodecidua from patients who had experienced labor than in those who had not had labor in term or preterm gestation. However, the expression of these chemokines and their receptors did not change between tissues from women in term gestation without labor and preterm gestation, except CXCL8, which was lower in tissues from women who delivered in term pregnancy without labor than in preterm gestation ($P = 0.025$) (Fig. 4).

CHORIODECIDUAL LEUKOCYTES EXPRESS SPECIFIC CAMs DURING BOTH TERM PREGNANCY AND SPONTANEOUS LABOR AT TERM

Cell adhesion molecules participate in leukocyte infiltration, spreading, and homing.[8,18,86,90] We therefore determined the expression of several CAMs in isolated choriodecidual leukocytes. Most of the CAMs were expressed in higher levels in tissues from patients in term gestation than in preterm gestation. Levels of *ICAM1* (intercellular adhesion molecule 1), *ICAM2*, *ICAM3*, *VCAM* (vascular cell adhesion molecule), *SELP* (selctin P), *ITGAL* (integrin alpha L), and *ITGAM* (integrin alpha M) were higher in tissues from patients in term gestation who had undergone spontaneous labor than in preterm gestation ($P \leq 0.025$). In addition, levels of *ICAM1*, *ICAM2*, *VCAM*, *SELP*, *ITGAL*, and *ITGAM* were greater in tissues from women who had not experienced labor in term gestation than in preterm gestation ($P \leq 0.025$). Only levels of *ITGAL* were higher in tissues from patients who had undergone spontaneous labor than in tissues from women who had not experienced labor in term gestation ($P = 0.05$) (Fig. 5).

CHORIODECIDUAL LEUKOCYTES EXPRESS HIGH LEVELS OF TNA-α, IL-1β AND MMP-9 DURING SPONTANEOUS LABOR AT TERM

Finally, the expression of TNF-α and IL-1β was determined in choriodecidual leukocytes. mRNA expression of *TNF-α*, *IL-1β* and *MMP-9* were higher in tissues from women who

had not experienced labor in term gestation than in preterm gestation ($P = 0.024$ each). *TNF-α* levels were also higher in tissues from patients who had undergone spontaneous labor than in tissues from women who had not experienced labor ($P = 0.05$) and greater in tissues from women who had not experienced labor in term gestation than in preterm gestation ($P = 0.024$). *IL-1β* levels in tissues from women who had not experienced labor in term gestation were higher than in preterm gestation. Although *MMP-9* levels did not change significantly between these two clinical groups, the mRNA levels of this enzyme did tend to increase in women at term pregnancy (Fig. 6a). Protein levels of TNF-α, IL-1β, and MMP-9 were also higher in tissues from women who had experienced labor than in those from women who had not experienced labor in term gestation. MMP-9 co-localized with IL-1β and TNF-α (Fig. 6b). MMP-9 was associated with leukocytes, including T cells, and this association was more evident in tissues form women who had experienced labor in term gestation (Fig. 7).

DISCUSSION

PRINCIPAL FINDINGS OF THE STUDY

(i) Memory-like $CD4^+$ T cells were present in the choriodecidual tissues of women at term without labor, as well as in women in spontaneous labor at term, indicating that cells involved in the adaptive immune response are present in the maternal-fetal interface at term. (ii) The proportion of 'memory-like T cells' is increased in the choriodecidual tissues of women in spontaneous labor. (iii) A new subset 'memory-like $CD4^-CD8^-$ double-negative T cells' are present in the choriodecidual tissues of women in spontaneous labor at term. (iv) Choriodecidual leukocytes, T cells and granulocytes, express chemokines and their receptors (e.g., CXCL8, CXCL10 and CXCR1-5), which we propose participate in the recruitment of these cells during spontaneous labor at term. (v) Choriodecidual leukocytes express specific cell adhesion molecules, which are likely to participate in the homing of these leukocytes into this specific anatomical site as term approaches and when labor occurs, (vi) Choriodecidual T cells and granulocytes isolated from tissues of women in labor express high levels of MMP-9, IL1β, TNF-α, mRNA, as well as protein. Such cytokines have been implicated in the initiation of labor at term (e.g., IL-1) and preterm as they stimulate prostaglandin production and exert other biological functions required for parturition.[91,103,108,109] MMP-9 has been implicated in the mechanisms of membrane rupture.[14,94–96,98,99,101,102,110] (vii) Collectively, the findings reported herein support a role for the adaptive limb of the immune response in the onset of spontaneous labor at term.

LABOR AS IN INFLAMMATORY PHENOMENON

Unbiased study of the tissues involved in the mechanism of spontaneous labor at term suggests that parturition is an inflammatory response.[1–13,15–22,28] Inflammation appears to play a key role in the common pathway of parturition based upon *in vivo* and *in vitro* experimentation (uterine contractility, cervical dilatation/repair, rupture of membranes, and detachment of the placenta and membranes.[103–105,111–116] Current consensus suggests that the maternal-fetal interface, choriodecidua, is the primary site where maternal leukocytes infiltrate and generate an inflammatory microenvironment during spontaneous labor at term.[9,10,15,17–22,35,82,86,117] This process is also important in uterine involution and cervical repair.[27,34,130,131]

The studies described in this communication were designed to investigate whether the number of leukocytes and discreet subpopulations change as a function of advance in gestational age and spontaneous labor at term. Therefore, we study women who had a preterm gestation, who were not in labor, but require a preterm delivery by cesarean section. Tissues from women not in labor at term were obtained from patients undergoing an elective

cesarean delivery before the onset of labor, and choriodecidua was collected from tissues of women who underwent normal parturition at term and received no medications. The key findings of this study are that the number of leukocytes present in the choriodecidual increases with gestational age and that there is a change in the proportion of different leukocytes in the choriodecidual tissues after labor has occurred.

A widely held belief for decades was that granulocytes played a key role in the onset of labor.[4,47,80] However, recent evidence in which mice have been depleted of neutrophil during pregnancy indicates that these cells are not necessary for the onset of labor in these species.[132] The same appears to be the case for monocytes/macrophages (Gomez-Lopez N, Bijland MT, Olson DM and Robertson SA, unpublished data). The data reported herein suggest that adaptive immune cells, and specifically T cells, play a role in the onset of spontaneous labor at term pregnancy. The relative contribution of the innate and adaptive cells to the process of parturition remains to be defined and it is being investigated by our group.

CHORIODECIDUAL T CELLS: MEMORY-LIKE CD4$^+$ T CELLS DURING TERM PREGNANCY AND SPONTANEOUS LABOR AT TERM

T cells have been localized in the choriodecidua in term pregnancies.[35,72,133–139] Choriodecidual T cells at term gestation seem to be activated and have both a regulatory and an effector phenotype.[35,134,135,139] Recently, we demonstrated that choriodecidual T cells are recruited into the maternal-fetal interface during spontaneous labor at term.[21,82] Here, we demonstrated that the proportion of choriodecidual T cells increases at term pregnancy, and it is maximal during spontaneous labor at term. To our knowledge, this is the first demonstration that the proportion of adaptive immune cells, T cells, increases during spontaneous labor at term pregnancy.

This study also showed that a high proportion of choriodecidual CD4$^+$ T cells express CD45RO (memory-like T cells) before and after spontaneous labor at term. The fact that these cells express CD45RO suggests that these T cells were generated from early pregnancy or even in a previous pregnancy.[140] It is tempting to postulate that memory T cells have T-cell receptors for paternal antigens. The adaptive immune system of the mother could have encountered these antigens in early pregnancy in the context of fetal transfusion of cells into the maternal circulation which occurs physiologically during pregnancy.[41,141–148] These cells have a full complement of class I and class II HLA, and there is evidence that mothers have cytotoxic T cells against paternal antigens even during normal pregnancy.[134,149–152]

A POSSIBLE ROLE FOR CHORIODECIDUAL MEMORY-LIKE CD4$^+$ T CELLS DURING SPONTANTEOUS LABOR AT TERM

Cells have been implicated in the tolerogenic state required during normal pregnancy (to avoid rejection of paternal antigens expressed in fetal tissues, such as white blood cells).[49,135,137,138,151,153] A role for effector T cells during labor was suggested approximately thirty years ago.[154] Yet, persuasive evidence of their participation in the mechanisms of parturition remains to be proven. We recently reported that T cells infiltrate the site of rupture of the fetal membranes (i.e., choriodecidua), and thus, they may play a role in spontaneous rupture membrane.[21,82] Here, we report that choriodecidual memory-like CD4$^+$ T cells express IL-1β, TNF-α and MMP-9 during spontaneous labor at term. These cytokines have been implicated in both term[10,17,91,109,155] and preterm labor[91,93,108,109,156]; however, the traditional thought is that these mediators are produced by cells of the innate immune system. We report for the first time that such mediators are also produced by cells of the adaptive immune system – T cells.

The generation of MMP-9 suggests that T cells may participate in the degradation of extracellular matrix of the fetal membranes and surrounding tissues.[4,14,94–96,98,99,101,102,110] T cells producing MMP-9 were recently identified in patients with multiple sclerosis, and they have been implicated in the pathogenesis of this disease.[157] Therefore, we propose that memory-like $CD4^+$ T cells producing MMP-9 and pro-inflammatory mediators participate in parturition during the process of spontaneous labor at term pregnancy.

Several studies have also demonstrated that maternal circulating T cells during pregnancy are the result of the expansion of the total number of regulatory T cells or Tregs.[140,153,158–162] Although the proportion of circulating regulatory T cells does not change in late gestation, the suppressive activity of these cells decreases during spontaneous labor at term.[161] We therefore suggest that the changes in functional properties of T regulatory cells, suppression, play an important role in the initiation of labor.[163] The role may not be restricted to spontaneous labor at term but they may also play an important role in preterm labor, particularly associated with maternal anti-fetal rejection.

A NOVEL FINDING: MEMORY-LIKE $CD4^-CD8^-$ DOUBLE-NEGATIVE T CELLS IN THE CHORIODECIDUAL INTERFACE DURING SPONTANEOUS LABOR AT TERM

Interestingly, 30% of $CD4^-CD8^-$ DN T cells are in the choriodecidua during spontaneous labor at term. They also seemed to express CD45RO; therefore, they were considered as memory-like T cells. Although DN T cells were previously reported,[164] here we demonstrated that their proportion increases during spontaneous labor at term. Human DN T cells are non-conventional T cells that show tropism for mucosa and behave more like innate rather than adaptive immune cells.[165] Human DN T cells act as either suppressors or promoters of an immune response. Their suppressor role in the effector function of T cells is mediated by an active cell contact-dependent mechanism. Human DN T cells can also produce pro-inflammatory cytokines and thus enhance the inflammatory response.[166,167] We suggest that the choriodecidual memory-like DN T cells may play both roles as they could suppress effector T cells (e.g., $CD4^+$ T cells) and/or help in the production of cytokines during spontaneous labor at term. More studies are needed to clarify their function.

HOW ARE MEMORY-LIKE T CELLS RECRUITED INTO THE CHORIODECIDUA DURING TERM PREGNANCY AND SPONTANEOUS LABOR AT TERM?

T-cell recruitment and homing are mediated by specific chemokines and CAMs. T-cell chemo-attractants include CCL5,[168] IL-16,[169] CXCL10, CXCL9, and CXCL11.[67,170, 171] Recently it has been demonstrated that early pregnancy decidual stromal cells restrict T-cell recruitment into the decidua to regulate the maternal immune response against the fetus.[172] Such findings suggest that the decidua has an active role in controlling the migration of maternal T cells into the maternal-fetal interface.[172,173] Our observations are in keeping with this hypothesis and suggest that both decidual stromal cells and leukocytes coordinate the migration of several immunological cells, including T cells, into the maternal-fetal interface all throughout pregnancy and during labor. We previously demonstrated that CCL5 (also known as RANTES) is expressed by the human fetal membranes (i.e., choriodecidua) during term pregnancy and that its local concentration does not change with labor.[19] However, *in vivo* observations suggest that RANTES increases in the amniotic fluid with spontaneous labor at term and with intra-amniotic infection/inflammation.[174] As CCL5 can recruit memory T cells[168] and its local concentrations in the choriodecidua increase during term pregnancy,[19] there seems to be a temporal association between the infiltration of memory-like $CD4^+$ T cells and the expression of a chemokine capable of recruiting these cells at term pregnancy. Therefore, we propose that CCL5 participates in the recruitment of memory-like $CD4^+$ T cells during term pregnancy, In addition, our data showed that

CXCL10 and its receptor *CXCR3* are highly expressed in choriodecidual leukocytes during spontaneous labor at term. This finding is consistent with our previous observation where we found that CXCL10 levels in the choriodecidual tissues are higher during labor than in the absence of labor.[19] As both levels of CXCL10 and the proportion of memory-like DN T cells increased during labor, we suggest that CXCL10 may be involved in memory-like DN T-cell recruitment during spontaneous labor at term. CXCL10 has been implicated in the mechanism responsible for maternal anti-fetal rejection and chronic chorioamnionitis, which is the most common lesion associated with spontaneous late preterm labor.[67] Therefore, the observations reported herein have implications beyond the control of normal parturition.

Cell adhesion molecules play a critical role in controlling adhesion and the homing of T cells into reproductive tissues.[8] It has been described in a murine model that ITGAL and ICAM1 mediate T-cell recruitment during pregnancy.[175] Here, we found that in human *ITGAL* and it ligands, *ICAMs 1-3* are over-expressed in choriodecidual leukocytes during term pregnancy and spontaneous labor at term. Because these CAMs have been related to the recruitment of human effector memory $CD4^+$ T cells,[176] we suggest that they may also be responsible for the infiltration and accumulation of memory-like T cells into the choriodecidua during term pregnancy and spontaneous labor at term.

WHAT IS THE ROLE OF GRANULOCYTES IN THE CHORIODECIDUA DURING SPONTANEOUS LABOR AT TERM?

Granulocytes are present in reproductive tissues during pregnancy.[4,9,10,13,177] The results of the present study indicate that granulocytes are present in the choriodecidua during late gestation. We found that although leukocytes are scarce in the choriodecidua before term pregnancy, most of them are granulocytes (~80%). These cells, and in particular neutrophils, have been sparingly found in the choriodecidua before the onset of labor.[10] Even though granulocytes were present approximately in 60% of leukocytes in the choriodecidua at term, they represented only 35% of leukocytes in tissues obtained in women who experienced labor. Our data and that of others, who have reported that decidual neutrophils in term pregnancies provide a rich source of extracellular matrix proteases[4,16,178–180] and cytokines,[12,33,74,80,92,181] suggest that granulocytes may play a role before and during spontaneous labor at term (before labor for the preparative stages for parturition). As the depletion of neutrophils in the mouse does not affect the timing or course of labor[132] and that other leukocytes and stromal cells produce these and other related immunological mediators,[83,182–188] it would seem that granulocytes are not absolutely required for labor in mice. We favor a model in which there is cooperation of the different cell types to achieve normal parturition at term.

WHAT FACTORS ARE IMPLCIATED IN THE RECRUITMENT OF GRANULOCYTES INTO THE CHORIODECIDUA DURING TERM PREGNANCY?

Neutrophil recruitment and homing are mediated by specific chemokines and CAMs. CXCL8 is highly expressed in the amniochorion and choriodecidua in spontaneous labor.[12,19,21,22,74,80,92,181] Coincidently, our results showed that *CXCL8* and it receptors, *CXCR1* ANC *CXCR2*, are greatly expressed in choriodecidual leukocytes during spontaneous labor at term pregnancy. Previous studies have associated neutrophil influx into decidua with CXCL8 levels.[12,19,21,22,74,80,92,181] Although we did not observe an increase in the proportion of granulocytes in the choriodecidua during spontaneous labor at term, we did observe that choriodecidual granulocytes express high levels of CXCL8 and its receptors, CXCR1 and CXCR2. This suggests that infiltration of granulocytes into the choriodecidua expresses CXCL8 as a labor mediator to promote labor at term, rather than to recruit granulocytes. An alternative could be that the choriodecidual leukocytes express high levels of CXCL8 to recruit more granulocytes, which will be required to repair surrounding tissue

(e.g., cervix) during the postpartum period.[131,132] CAMs like selectin E, VCAM-1, and ICAM-1 have been linked to neutrophil recruitment into reproductive tissues.[13,110] We put forward evidence supporting this hypothesis as our data showed that *VCAM* and *ICAM1* are highly expressed at term pregnancy, where granulocytes are abundant in the choriodecidua. Taken together, these data allow us to suggest that the synchronized action of CXCL8 produced by fetal membranes and choriodecidual leukocyte expression of CXCL8 receptors and CAMs (e.g., VCAM and ICAM-1) results in the recruitment and homing of granulocytes into the choriodecidua during term pregnancy and spontaneous labor.

STRENGTHS AND LIMITATIONS

This is the first study identifying adaptive immune cells, memory-like $CD4^+$, and double-negative T cells, which are able to produce MMP-9 in the choriodecidua during spontaneous labor at term pregnancy. We recognized the following limitation: (i) the identification of memory T cells is very complex and uses the expression of several markers including CD45RO, CD45RA, CCR7, CCR4, CCR5, CD62L, CD27, CD28, CXCR5, CXCR3 and CRTH2.[189] In our study, we only analyzed the expression of CD45RO and CD45RA; therefore, we referred to these cells as 'memory-like T cells' and further analysis of their phenotype is required; (ii) additional studies are necessary to clarify the role of choriodecidual memory-like $CD4^+$ T cells during term pregnancy and spontaneous labor at term; (iii) the function of double-negative T cells in the choriodecidua during spontaneous labor at term remains to be elucidated; (iv) choriodecidual granulocytes include neutrophils, basophils and eosinophils; therefore, future studies are needed to establish the proportion of each granulocyte and their possible role during term pregnancy and spontaneous labor at term. The tissues obtained from women with preterm gestations were derived from patients who had complications such as congenital anomalies. It is possible that these patients do not represent the state of the decidual in normal pregnancy. However, it is extremely difficult in humans to obtain preterm tissues in normal women; therefore, such tissues are the best representation that can be obtained at this time of the cellular composition of the choriodecidual in preterm pregnancy.

CONCLUSION

Human labor involves the establishment of an inflammatory microenvironment in the choriodecidua that includes adaptive immune cells, such as two newly identified T-cell subsets, 'memory-like $CD4^+$ T cells' and 'memory-like double-negative T cells'. This microenvironment also includes granulocytes, macrophages and an extensive signaling network composed of chemokines, cytokines and cell adhesion molecules. We propose that cells and mediators create a specific microenvironment in the maternal-fetal interface that plays an important role in spontaneous labor at term pregnancy (Fig. 8). Further research is needed to investigate the function of these T cells during term and preterm labor. An improved understanding of the mechanisms of term and preterm labor will assist in the prevention of prematurity, the most important challenge to modern obstetrics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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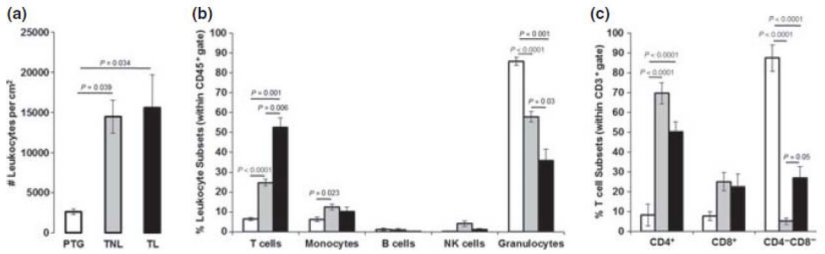

Figure 1.

Number and phenotype of choriodecidual leukocytes. PTG, white; TNL, gray; TL, black bars. (a) Number of leukocytes per cm^2 of tissue. Leukocyte density increased at term of pregnancy. (b) Leukocyte subsets were analyzed within the $CD45^+$ gate. While T cells increased, granulocytes decreased from PTG to TL. Monocytes were greater in TNL than in PTG. (c) T-cell subsets were analyzed within the $CD3^+$ gate. TNL and TL included a high proportion of $CD4^+$ T cells. $CD3^+CD4^-CD8^-$ DN T cells were higher in TL cells were higher in TL than in TNL. Data shown are means ± SEM, with five to seven tissues per group.

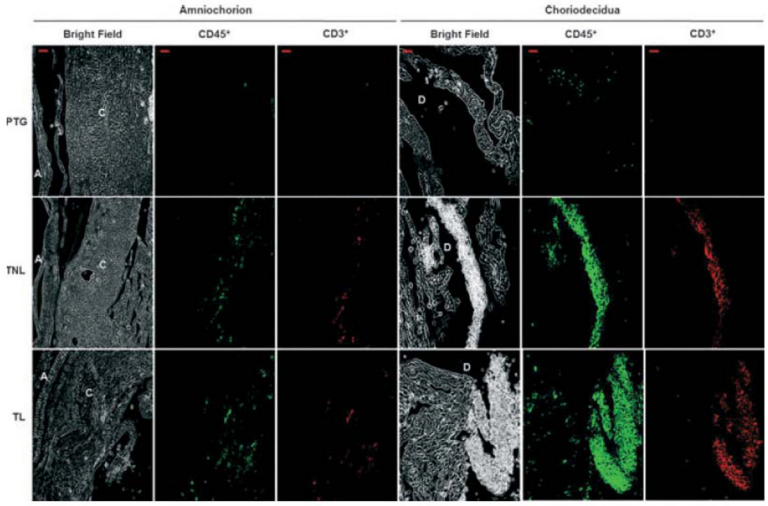

Figure 2.

Tissue localization of choriodecidual leukocytes. Photomicrograph of amniochorion (left panel) and choriodecidua (right panel) in each group. A, amnion; C, chorion; D, decidua. Leukocytes ($CD45^+$) were greater in choriodecidua than in amniochorion, and their density seemed to increase from PTG to TL. T cells ($CD45^+CD3^+$) and granulocytes ($CD45^+CD3^-CD14^-CD56^-CD19^-$) increased from PTG to TL. Bar, 20 μm. Confocal microscopy: magnification x200. Data are representative of three or more independent experiments with five tissues per group.

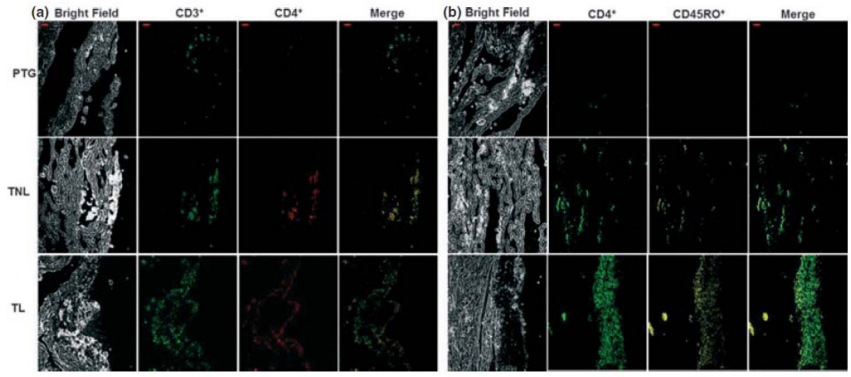

Figure 3.

Tissue localization of $CD45RO^+$ memory-like T cells. (a) Photomicrograph of choriodecidua identifying $CD4^+$ T cells in each group. (b) Photomicrograph of choriodecidua identifying $CD4^+$ $CD45RO^+$ memory-like T cells in each group. Merge shows the co-localization of both markers. $CD45RO^+$ memory-like T cells seemed to increase from PTG to TL. Bar, 20 μm. Confocal microscopy: magnification x200. Data are representative of three or more independent experiments with five tissues per group.

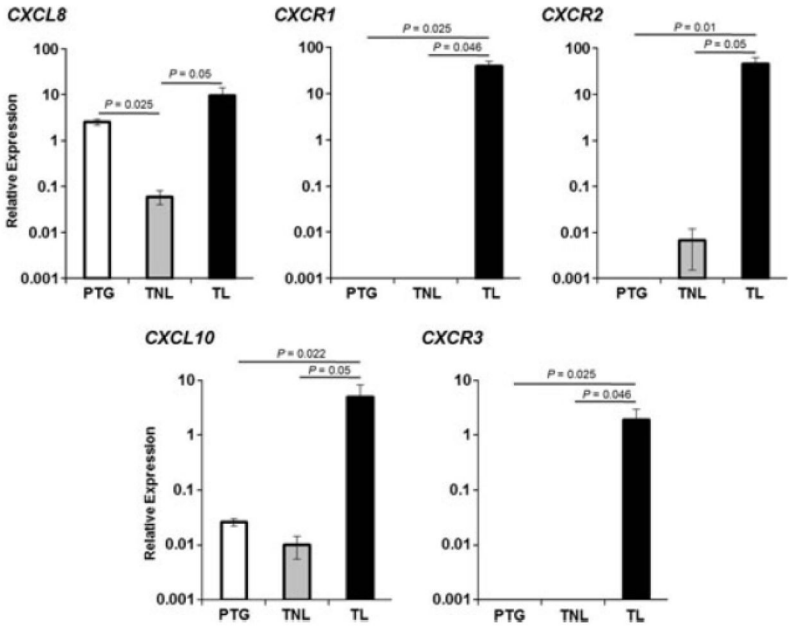

Figure 4.

Chemokine expression. Relative expression of *CXCL8* and *CXCL10* and its receptors *CXCR1-3* in choriodecidual leukocytes from each group. *CXCL8* was higher in TL than in TNL, and lower in TNL than in PTG. *CXCL10*, *CXCR1*, *CXCR2*, and *CXCR3* were higher in TL than in TNL and PTG. Data are expressed as relative expression using *ACTB* gene as the reference. Data shown are means ± SEM, with five to seven tissues per group.

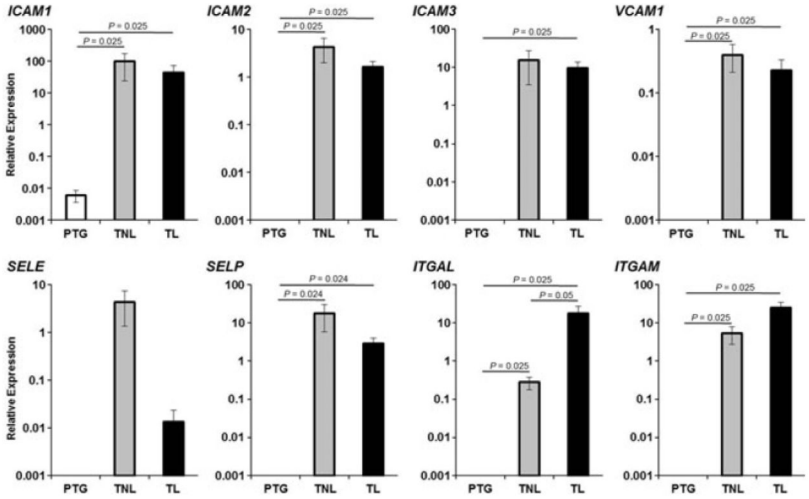

Figure 5.

Cell adhesion moelcules expression. Relative expression of *ICAM1, ICAM2, ICAM3, VCAM, SELP, SELE, ITGAL* and *ITGAM* were higher in TNL than in PTG. *ITGAL* was higher in TL than in TNL. Data are expressed as relative expression using *ACTB* gene as the reference. Data shown are means ± SEM, with five to seven tissues per group.

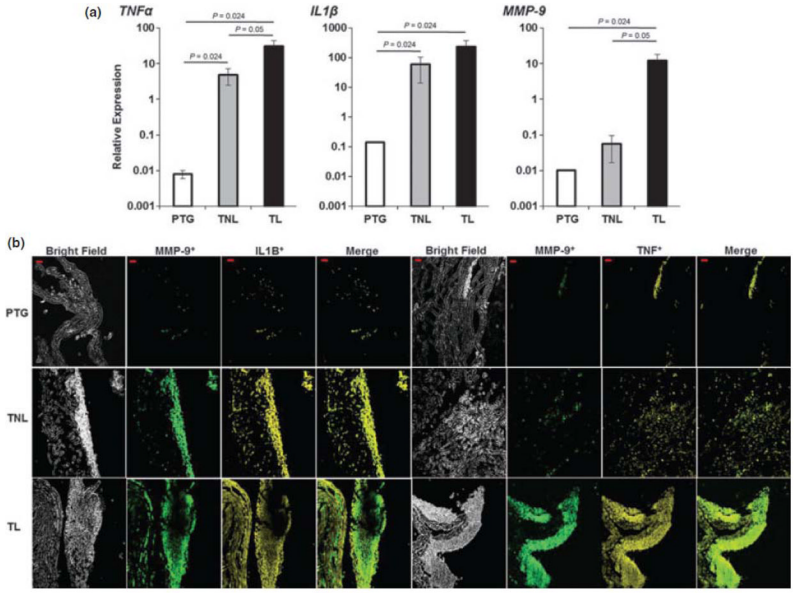

Figure 6.

Labor mediators. (a) Expression of labor mediators. *IL-1β, TNF-α, and MMP-9* in choriodecidual leukocytes from each group. All three mediators increased from PTG and TL. Data are expressed as relative expression using *ACTB* gene as the reference. Data shown are means ± SEM, with five to seven tissues per group. (b) Photomicrograph of choriodecidua from each group. IL-1β, TNF-α, and MMP-9 increased from PTG to TL. Merge shows the co-localization of both markers. Bar, 20 μm. Confocal microscopy: magnification x200. Data are representative of three or more independent experiments with five tissues per group.

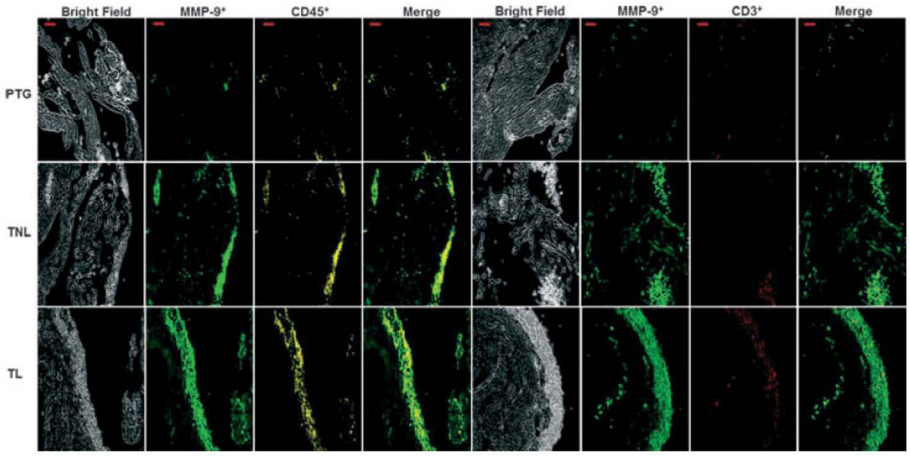

Figure 7.

Localization of MMP-9 in choriodecidual leukocytes. Photomicrograph of choriodecidua identifying MMP-9^{+}CD45^{+} cells or MMP-9^{+}CD3^{+} T cells in each group. Both MMP-9^{+} total leukocytes and T cells increased from PTG to TL. Merge shows the co-localization of both markers. Bar, 20 μm. Confocal microscopy: magnification x200. Data are representative of three or more independent experiments with five tissues per group.

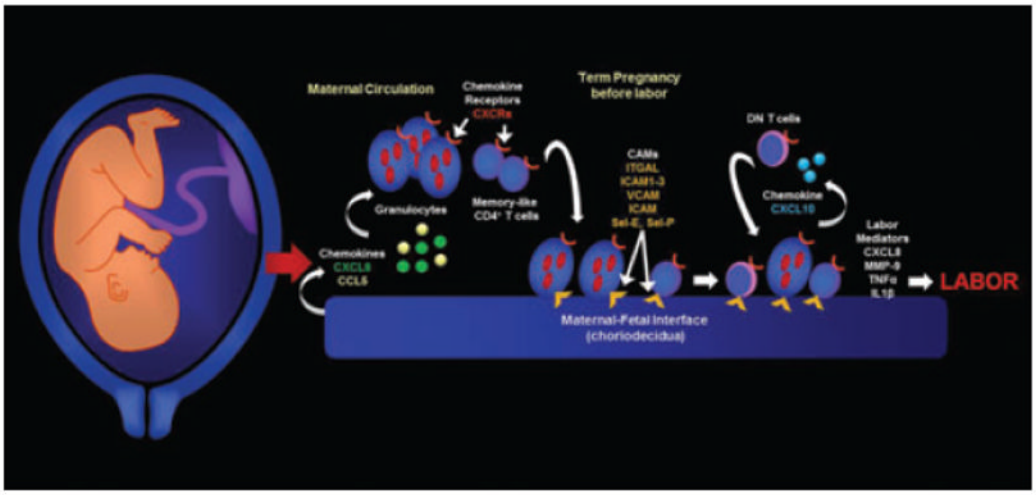

Figure 8.

Conceptual framework. Choriodecidual tissues express chemokines to recruit maternal circulating leukocytes at term pregnancy. Before the onset of labor, choriodecidual memory-like $CD4^+$ T cells and other leukocytes such as granulocytes express cell adhesion molecules to remain into this anatomical space. Infiltrating leukocytes release CXCL10 to recruit double-negative (DN) T cells. Together they release labor mediators (e.g., CXCL8, IL-1β, TNF-α and MMP-9) to participate in labor at the end of gestation.