Activation of peripheral leukocyte migration before spontaneous labor at term

OBSTETRICS

Activation of peripheral leukocyte migration before spontaneous labor at term

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BACKGROUND: Leukocytes are induced to migrate into the uterus at parturition, releasing cytokines and chemokines that activate it for delivery. A specific chemotactic signal is required for these actions, and published evidence suggests that it comes from the human fetal membranes and has a time-dependent component (ie, cells obtained at term in labor migrate more than cells obtained at term not yet in labor). The hypothesis that the fetal membrane chemoattractants activate the leukocytes to become responsive for migration was tested.

OBJECTIVE: This study aimed to: (1) examine the changes in leukocyte migration-responsiveness longitudinally from the late third trimester, to in labor, to 3 days postpartum; (2) explore the specific week-to-week changes in migration before delivery; (3) define the timing of chemokine receptor expression patterns in leukocytes relative to migration and the changes in cytokine and chemokine concentrations in maternal serum; (4) examine the ability of term fetal membrane-conditioned medium and term maternal serum to increase cell responsiveness; and (5) test the potential of the leukocyte migration assay to predict delivery within 1 week.

STUDY DESIGN: Leukocyte migration in response to a chemoattractive extract of term human fetal membranes was studied using a modified Boyden chamber. Flow cytometry assessed migrated cell phenotypes. The relationship between the expression of chemokine receptors and migration was tested using quantitative polymerase chain reaction, the bioassay, and regression analyses. Cytokines and chemokines in maternal serum were quantified using multiplex analysis. Conditioned medium from fetal membrane explants and maternal serum were evaluated for their abilities to enhance leukocyte migration using the bioassay. The ability of the bioassay to predict term delivery was assessed using receiver-operating characteristic curve and cost-curve analysis.

RESULTS: The number of leukocytes that migrated at term delivery was increased relative to the late third trimester, followed by a significant fall in numbers that migrated at 3 days postpartum (P=.002). The largest increase in migrated cells occurred 1 to 2 weeks before delivery. The messenger RNA abundance of several chemokine receptors increased in peripheral leukocytes at term in labor relative to the third trimester, and this correlated with an increase in migrated cells in 5 of 6 cases (R=0.589 to 0.897; P<.03). The concentrations of several chemokines and cytokines in maternal serum increased with labor onset. Fetal membrane explant-conditioned medium and maternal serum obtained at term labor increased the responsiveness of leukocytes to fetal membrane chemoattractive extract. The bioassay was demonstrated to predict delivery within 7 days with excellent performance characteristics using a cohort prevalence of 71.7% (positive predictive value=96.1%; negative predictive value=58.5%; sensitivity=74.2%; specificity=92.3%; positive likelihood ratio=9.25; and negative likelihood ratio=0.28). A single determination was validated to have a high degree of confidence.

CONCLUSION: Term human fetal membranes release chemoattractants near the end of pregnancy that increase in ability to activate and attract an increasing number of leukocytes as gestation advances.

Key words: biomarker, chemoattractants, chemokines, common pathway of parturition, cytokines, delivery, fetal membranes, maternal serum, neutrophils, prediction, pregnancy

Introduction

Leukocytes invade the uterus at various times throughout pregnancy to facilitate events such as implantation, uterine involution, and postpartum cervical remodeling.[1−3] Histologic evidence shows that peripheral circulating leukocytes invade human and rodent gestational tissues to effect labor for both term and preterm birth (PTB).[4−12] Neutrophils, macrophages, and T-lymphocytes invade the human myometrium and decidua before and during term labor,[6,7,10,11] and leukocytes infiltrate the cervix at term.[4,5,8,9] Once positioned in these tissues, it has been proposed that these cells release many types of proinflammatory effectors that contribute to an inflammatory event that facilitates uterine activation for labor (eg, altered expression of uterine activation proteins, remodeling of the cervical extracellular matrix, and breakdown of the fetal membranes).[3,12−15]

Enhanced migration of human leukocytes at delivery was first demonstrated ex vivo in a chemotactic assay using a nonspecific chemoattractive extract.[16] Our collaborators and we identified that the human fetal membranes (hFM) (amnion and chorion with attached decidua vera) release chemoattractants whose potency increases as term labor approaches, as evident from hFMs obtained from women at term not in labor and women following term vaginal delivery. The peripheral circulating leukocytes' responsiveness to these chemoattractants also increases as labor approaches.[17−20] We used the published leukocyte migration assay (LMA), where leukocytes in an upper

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AJOG at a Glance

Why was this study conducted?

Maternal leukocytes invade the uterus before term delivery to promote the physiology of birth. We examined the pattern of migration, ex vivo, and studied how they become activated.

Key findings

Peripheral leukocytes increase their migration 1 to 2 weeks before term delivery in response to chemoattractants produced by the fetal membranes. These fetal membrane chemoattractants increased the responsiveness of the leukocytes and attracted them in our bioassay. Maternal serum also increased the migration-responsiveness of the leukocytes, suggesting that fetal membrane chemoattractants enter maternal blood.

What does this add to what is known?

The gradual then steep weekly increase 1 to 2 weeks before delivery in ex vivo leukocyte migration is described along with the sharp decrease 3 days postpartum after delivery of the placenta and fetal membranes. The data suggest that the source of the factor(s) that activate and attract the leukocytes are the fetal membranes.

chamber are stimulated to migrate through a filter with tiny pores to the bottom chamber by a chemoattractive extract obtained from the hFMs. This assay mimics some of the in vivo actions of leukocyte extravasation and invasion, and we have used it to study this phenomenon in rats, guinea pigs, mice, and humans at term and preterm.[14,19–21]

The purpose of this study was to understand the patterns and regulation of leukocyte migration during late human pregnancy and at term labor and delivery using the LMA as a bioassay. We also explored if the LMA could predict the timing of term labor in humans. Our primary hypothesis was that there is an increase in leukocyte migration at term delivery and that the chemoattractants released by hFMs regulate this leukocyte behavior. Our secondary hypothesis was that the bioassay could predict term delivery following labor within 7 days.

Materials and Methods

Participants

Blood collection for measuring leukocyte migration during the final weeks of pregnancy and postpartum

This study was performed at Juntendo University (Tokyo, Japan). Eleven recruited pregnant women satisfied the inclusion and precise sampling criteria for the longitudinal study that involved collecting a peripheral blood sample at a specified moment in pregnancy and assaying the leukocytes for migration immediately after collection. The inclusion criteria were patients with normal singleton pregnancies without medical or obstetrical complications. The exclusion criteria were women with a clinical infection, premature rupture of membranes, diabetes mellitus, immunologic problems, nonsingleton pregnancies, fetal congenital diseases, intrauterine growth restriction, preeclampsia or dysfunctional labor, and recipients of progesterone or artificial oxytocin. Blood was drawn from patients at 3 time points, including during a routine health checkup at the 35th or 36th week of gestation, when labor started with the first stage of delivery, and on postpartum day 3.

Blood collection for assessing weekly changes in leukocyte migration immediately before term labor

An additional 6 women were recruited from the Royal Alexandra Hospital in Edmonton, Alberta, Canada, with the same inclusion and exclusion criteria as above. Blood was collected weekly by our research nurse over the last 3 to 5 weeks of gestation, and the migration potential of its leukocytes was assessed using the LMA. These data provided more precise information regarding week-to-week changes in migration in late gestation in individual participants.

Blood collection for ex vivo studies: adaptive changes in leukocytes

Human whole blood was collected from preterm not in labor (PNL) (28−36 weeks), term not in labor (TNL), and term in labor (TL) women at the Royal Alexandra Hospital. Labor was defined by a cervical dilation of ≥4 cm in the presence of uterine contractions. The same inclusion and exclusion criteria as above were used.

Blood collection for assessing the leukocyte migration assay diagnostic performance at term labor

A total of 93 pregnant but not in labor women at the First Affiliated Hospital of Chongqing Medical University (Chongqing, People's Republic of China) were recruited during the late third trimester between 37 weeks, 3 days and 41 weeks, 0 days of gestation. Each participant provided a 4-mL blood sample for the LMA at that time. Days later, they all delivered by spontaneous vaginal delivery or via cesarean delivery after the onset of labor. The inclusion and exclusion criteria were the same as above.

Placental collection for fetal membrane isolation

The TNL hFMs were prepared from placentas collected from women undergoing an elective cesarean delivery at the Royal Alexandra Hospital. The TL hFMs were prepared from placentas collected (at all 3 sites: Japan, China, and Canada) from women who underwent spontaneous vaginal delivery at term. The same inclusion and exclusion criteria described above were followed.

Methods

Isolating leukocytes for the leukocyte migration assay

The LMA requires leukocytes from maternal peripheral blood.[21] Peripheral blood (4 mL) was collected from pregnant women into tubes with heparin coating, and 0.8 mL HetaSep

(STEMCELL Technologies, Tokyo, Japan) was added to separate the leukocytes from the erythrocytes. Solutions were mixed well, and the tubes were placed in an incubator at 37°C for 10 minutes to allow the sample to settle until plasma separation. The leukocyte-rich plasma layer was transferred to a 50-mL tube and washed with a 4-fold volume of 1× phosphate-buffered saline (PBS). The sample was centrifuged at 120×g for 10 minutes at 20°C to remove platelets. The supernatant was removed, and leukocytes were resuspended in 4 mL HyClone Roswell Park Memorial Institute 1640 medium (RPMI) (Sigma-Aldrich, St. Louis, MO) containing 2.0 mM L-glutamine and sodium bicarbonate. Leukocytes were counted using KOVA Glasstic Slide 10 with Grids (KOVA International Company, Biochemical Diagnostics Inc., Garden Grove, CA). Dead leukocytes were measured using the trypan blue method, and the suspension mixture was used if the viability rate was >95%. The leukocyte suspension was diluted with RPMI to a final concentration of 1×10^5 cells/50 μL and was used in the LMA within an hour of isolation.

Preparing fetal membrane extracts for the leukocyte migration assay

Fetal membranes were isolated and prepared from TL or TNL placentas, as previously described.[17,21] Immediately after delivery, hFMs were cut into 5 cm square pieces and washed 3 times with PBS to remove blood and debris. The pieces were minced with scissors and homogenized with 2 mL of Dulbecco's Modified Eagle Medium (DMEM) and then centrifuged at 3000×g for 30 minutes at 4°C followed by 20,000×g for 1 hour at 4°C. Supernatants were collected and pooled, and the protein concentration of each sample was measured using the bicinchoninic acid method and then adjusted to 4 μg/μL with DMEM.[22] Each sample was examined for chemoattractant activity toward leukocytes using the LMA. Fetal membrane extracts from 80 women were combined, aliquoted into 100 μL samples in vials, and stored at −80°C. A single vial was thawed for use in each LMA.

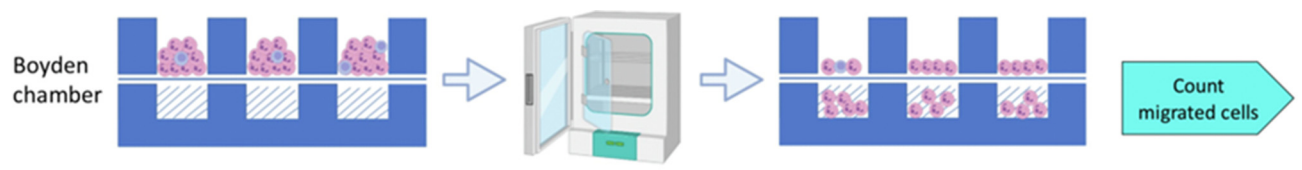
Leukocyte migration assay protocol

Modified Boyden chemotaxis chambers (AP48; Neuro Probe, Gaithersburg, MD)[21] or 3-dimensional printed chambers were used for the LMAs. Figure 1 depicts the Boyden chamber apparatus. The wells are divided into upper and lower compartments separated by a polycarbonate membrane (3-μm pores) (Neuro Probe) that is held in place with a rubber gasket. We placed the thawed hFM extracts (25 μL) or DMEM as a negative control in the lower compartments. Isolated leukocytes from pregnant women (100,000 or 200,000) are placed in the upper compartment of each well. The chemotaxis chambers were incubated for 90 minutes at 37°C in a humidified air incubator containing 5% CO_2. After incubation, the liquid in the lower compartment containing the migrated leukocytes was removed and placed into 5-mL Falcon polystyrene round-bottom tubes (Thermo Fisher Scientific K.K., Yokohama, Japan). OptiLyse (Beckman Coulter, Brea, CA) (300 μL) was added to lyse the red blood cells, and the sample was vortexed immediately for 1 second. The tube was incubated for 15 minutes at room temperature (20°C–25°C) in the dark. PBS (1 μL) was added to each tube, followed by centrifugation at 1000×g for 10 minutes at 4°C. After removal of the supernatant, 475 μL of PBS with 1% formalin was added to resuspend and fix the migrated leukocytes. These samples were then covered and stored at 4°C until analysis with flow cytometry.

Quantifying leukocyte migration with flow cytometry

We assessed the types of leukocytes that migrated from the upper compartments to the lower compartments with flow cytometry. Leukocytes were identified using BD FACSVerse (BD Biosciences, Tokyo, Japan) with CountBright Absolute Counting Beads (Life Technologies, Tokyo, Japan) (25 μL), which equates to 27,000 beads added before analysis. At least 10,000 events were collected for all cells, and data were saved for later analysis on FlowJo (FlowJo LLC, Ashland, OR). The flow cytometer was set to analyze the samples for 30 seconds, which involved 0 to 5000 events, and data were collected using BD FACSDiva

FIGURE 1
Flow diagram of the LMA



Chemoattractant (25 μL) from the supernatant of term homogenized human fetal membranes is pipetted into the lower compartment of the Boyden chamber, and 100,000 or 200,000 isolated leukocytes in 100 μL buffer are pipetted into the upper compartment. They are separated by a polycarbonate filter with 3-μm pores. During incubation for 90 minutes at 37°C, the cells migrate through the filter to the bottom. Following incubation, the cells in the bottom compartment are collected and quantified by flow cytometry. Figure designed using BioRender.

LMA, leukocyte migration assay.

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software (BD Biosciences). Leukocytes were gated on the basis of their forward (FSC) and side scatter (SSC). The total number of leukocytes migrated was calculated using the number of beads added to the tube multiplied by the ratio of leukocytes to beads in the sample aliquot taken by the flow cytometer. The number of leukocytes that migrated in the negative control wells containing DMEM (ca 50−100 cells) was subtracted from all samples in the final calculations.

Effect of maternal serum on leukocyte responsiveness

The ability of TL or TNL maternal serum to enhance the LMA responsiveness of TNL leukocytes was tested. TL or TNL whole blood was left to clot at room temperature for 30 minutes. The serum was separated by centrifugation at 1500×g for 10 minutes at 4°C, collected, and then stored immediately at −20°C. TNL leukocytes (1×10^7) were incubated with the serum in a 50:50 ratio for 1 hour at room temperature under gentle agitation using a stir bar. The leukocytes were then isolated via density centrifugation and resuspended in RPMI, and their migration was assessed using the LMA.

Leukocyte incubation with human fetal membrane−conditioned medium

The ability of TL or TNL hFM secretions to enhance the responsiveness of TNL leukocytes was also tested. hFM explants from TNL and TL placentas were excised using a 6-mm tissue punch and washed with Hanks' balanced salt solution.[23] Explants were plated in a 12-well transwell plate (Corning Life Sciences, Tewksbury, MA) with the chorion facing down. The transwells were filled with DMEM F-12 (HyClone, GE Healthcare LIfe Sciences, Missisauga, Canada) containing 15% fetal bovine serum and 1× antibiotic/antimycotic (100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B; HyClone, GE HealthCare Life Sciences, Mississauga, Canada). Explants were first acclimatized for 48 hours at 37°C and 5% CO_2. The medium was replaced and collected after a 24 hour incubation (conditioned).

Leukocytes (from TNL and TL women) were incubated with TL or TNL explant-conditioned media for 1 hour at 37°C with gentle agitation using a stir bar to prevent clumping. The leukocytes were pelleted and resuspended in RPMI, and their responsiveness was assessed using the LMA.

Real-time quantitative reverse transcription polymerase chain reaction

Expression patterns for key gene products related to cell migratory events were examined using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Four milliliters of each blood sample was lysed using red blood cell lysis buffer (BOSTER, Pleasanton, CA) according to the manufacturer's instructions. RNA was extracted from the red blood cell−free samples using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. RNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) to measure the optical density (OD) photometrically at 280 and 260 nm (an $OD_{260nm/280nm}$ ratio of >1.8 was considered to indicate protein-free RNA).[24] Primers for the *CCR* (C-C motif chemokine receptor) *1* to *7* and *CXCR5* (C-X-C motif chemokine receptor 5) genes were designed using the National Center for Biotechnology Information's Primer Blast. An annealing temperature of 60°C was used for all primers. Quantitative gene expression analysis was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) using SYBR Green Master Mix (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. Melt curve analysis was performed to ensure that the amplification of nonspecific products did not occur. Target gene levels were expressed relative to *ACTB* (β actin).

Statistical analysis

Data were tested for normal distribution using the Kolmogorov−Smirnov test, and the square root transformation was applied to the data to alleviate deviation from normal distribution if required. The longitudinal estimates of leukocyte migration from participants were analyzed using analysis of variance (ANOVA) for repeated measures (RM-ANOVA) to test for changes in the grouped individual data over time (Figure 3) and by regression analysis (Figures 3, B and 4). The data in Figure 3, B (number of cells migrated) were converted to ratios of the first observation, identified as 1.0. When a significant F value was obtained, post hoc paired *t*-tests were used to determine differences of means from individual times. In other figures, data were analyzed using 1-way ANOVA followed by the Tukey multiple-comparison post hoc test when a significant F value was determined. Data are shown as the mean±SEM; a *P* value ≤.05 was considered significant. The receiver-operating characteristic curve analysis was performed according to the method of Greiner et al[25] using this tool.[26] We used cost-curve analysis to assess the degree of confidence that a single determination of the LMA provided. The cost-curve analysis was performed by Dr Robert Holte, Department of Computing Science, University of Alberta.[27]

Study approval

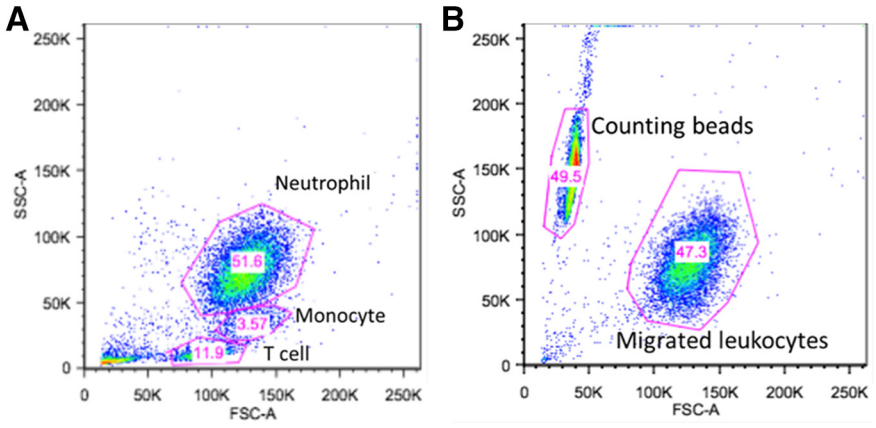
Regardless of the location, all samples were collected from women after informed written consent. The Japanese study was approved by the local ethics committee of Juntendo University, Faculty of Medicine (No.13-131). The samples collected at the Royal Alexandra Hospital were approved by the Institutional Review Board at the University of Alberta (Pro00069209 and Pro00060947). The Institutional Review Board at the First Affiliated Hospital of Chongqing Medical University approved their portion of the study.

Results

The migrating cells are nearly exclusively neutrophils

Figure 2 describes a flow cytometry analysis of the relative proportion of isolated leukocytes loaded into the upper compartment in a typical LMA (Figure 2, A) and the cells that migrated across the filter toward the hFM extracts (Figure 2,

FIGURE 2
Neutrophils comprise 99% of the migrated cells



A, Flow cytometry using antibodies against cell surface protein markers that, in combination, identify specific leukocytes (CD11b, CD14, CD3, and CD4) and specific gates for each leukocyte population shows the relative proportions of leukocytes isolated from term-labor women placed into the upper Boyden compartment. **B,** 99% of cells that migrated to the lower compartment were positive for CD11b.

FSC-A, forward scatter, channel A; *SSC-A*, side scatter, channel A.

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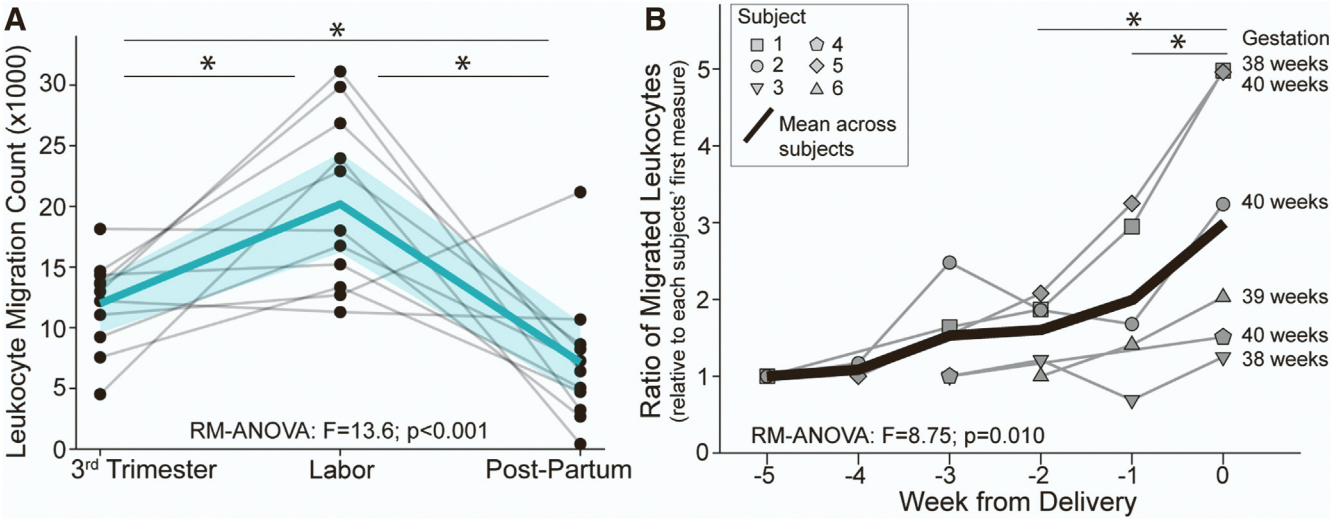
B). A subset of samples was measured by flow cytometry using antibodies (CD11b, CD14, CD3, and CD4) to distinguish leukocyte cell types. Approximately 99% of migrated leukocytes were neutrophils due to CD11b expression and lack of expression of the other markers, which were detected by setting specific gates for each leukocyte population based on dot-plot graphs of SSC vs FSC or detecting cells positive for CD11b+.

Leukocyte migration during the final weeks of pregnancy and postpartum

We studied the leukocyte migration-responsiveness of 11 participants (age range, 29–42 years) at 3 different time points, including in the third trimester (average: 36 weeks, 1 day of gestation), at TL (average: 39 weeks, 4 days), and postpartum (all at 3 days) (Figure 3, A). An RM-ANOVA test was performed and showed that significant differences exist

FIGURE 3
Longitudinal migration of leukocytes during the final trimester, at delivery, and 3 days post-partum, and during the final 5 weeks of pregnancy



A, Longitudinal mean migration values in the same 11 participants over 3 different time points (third trimester, labor, and 3 days postpartum) demonstrate that significant differences in migration rates exist between the 3 time points (RM-ANOVA, F=13.6; $P<.001$). Post hoc paired *t* tests to compare values between the 3 time points were all significantly different: third trimester vs labor ($P=.004$), labor vs 3 days postpartum ($P=.002$), and third trimester vs 3 days postpartum ($P=.03$). *Asterisk* denotes $P<.05$. **B,** During the final 5 weeks of pregnancy, the ratio of migrated leukocytes (calculated relative to each participant's first measure) becomes significantly greater than 1.0 by 2 weeks from delivery. We tested for differences using an RM-ANOVA over the last 3 time points, which were significant (F=8.75; $P=.01$). Post hoc pairwise comparisons indicated that delivery was significantly different from days −1 ($P=.012$) and −2 ($P=.043$); however, days −1 and −2 were not significantly different from each other. The "mean across subjects" line partially predicts the migration of cells (R=0.589; $P=.009$). *Asterisk* denotes $P<.05$.

RM-ANOVA, repeated measures analysis of variance.

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FIGURE 4

Leukocyte chemokine receptor expression and correlation with migration

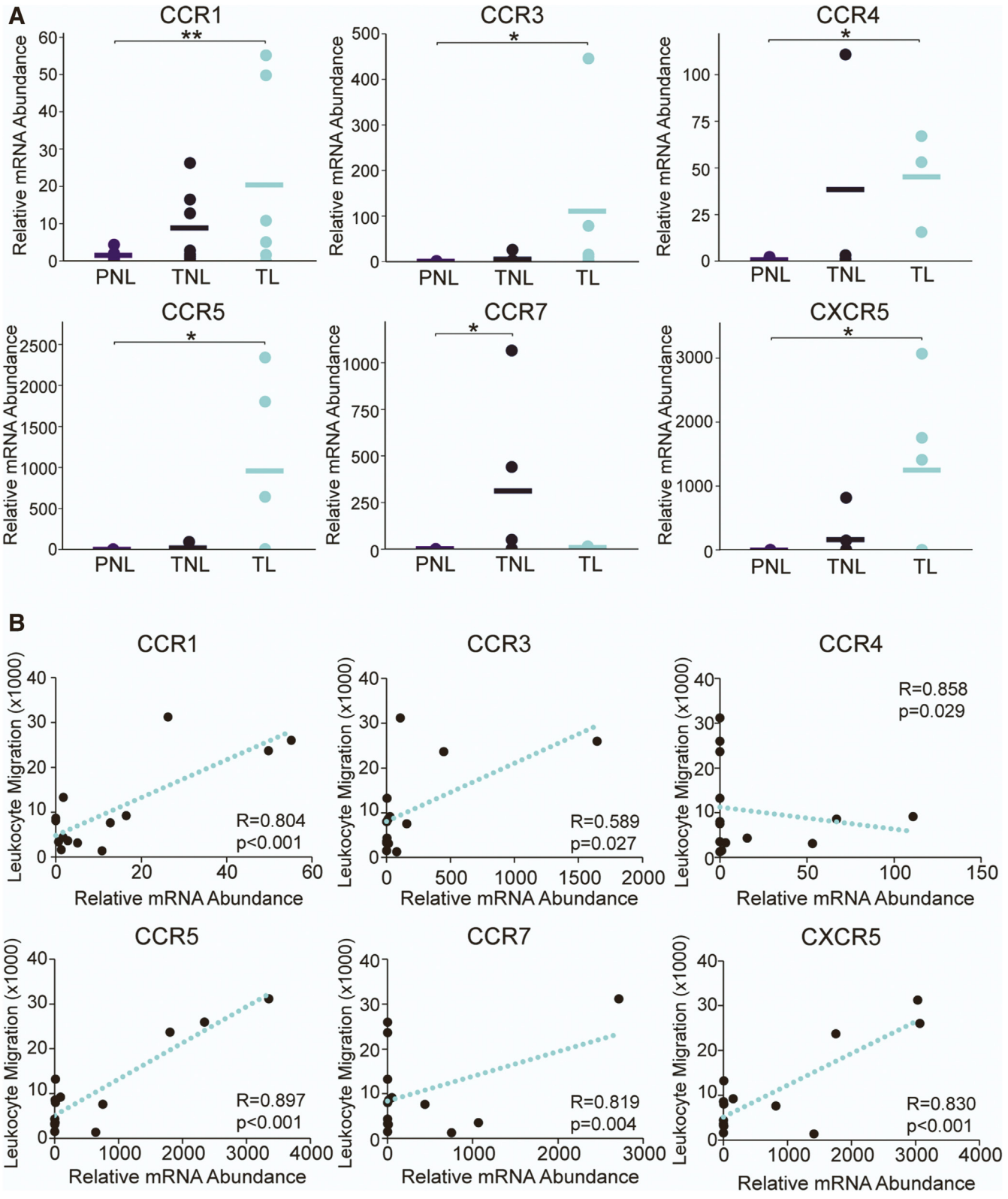

TABLE
Characteristics of chemokine receptors associated with leukocyte migration toward term human fetal membrane chemoattractants

Gene symbol	Expression on leukocytes	Major chemokine ligands	Function	Ref.
CCR1	Neutrophils, monocytes, T-cells, basophils, natural killer cells, mast cells	CCL3, CCL5, CCL8	Chemotaxis	Sokol and Luster, 2015
CCR3	Eosinophils,[a] T-cells, basophils	CCL11, CCL24	Chemotaxis, allergic reactions	Gerber et al,[29] 1997
CCR4	T-cells	CCL17, CCL22	Chemotaxis	Kunkel et al,[30] 2002
CCR5	T-cells	CCL3, CCL5	Chemotaxis	Kunkel et al,[30] 2002
CCR7	T-cells, B-cells	CCL19, CCL21	Chemotaxis	Olson and Ley,[31] 2002
CXCR5	B-cells, CD8+ T-cells	CXCL13	Chemotaxis	Pan,[32] 2022

CCL, C-C motif chemokine ligand; *CCR*, C-C motif chemokine receptor; *CXCL13*, C-X-C motif chemokine ligand 13; *CXCR5*, C-X-C motif chemokine receptor 5.

[a] Dominant expressing cells.

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in leukocyte migration rates between these 3 time points (F=13.6; $P<.001$). Leukocyte migration-responsiveness peaked at TL compared with the third trimester ($P=.005$). However, we observed a drop at 3 days postpartum ($P=.002$) to levels significantly lower than in the third trimester ($P=.03$).

To provide more detailed data about the week-to-week changes in leukocyte migration up to term delivery, we studied the longitudinal changes in the migration of leukocytes from 6 women during the last 2 to 5 weeks of pregnancy to TL hFM chemoattractive extracts (Figure 3, B). The data are described as incremental ratio changes from the previous week. We observed significantly higher ratios of migrated leukocytes at delivery than during weeks −2 ($P=.043$) and −1 ($P=.012$), and the largest increase in migration occurred between −1 week and delivery.

Leukocyte chemokine receptor expression increases with term labor and correlates with migration

Assuming that an increase in migration at term would be due to increased responsiveness to chemokines, we assessed whether 6 chemokine receptors demonstrated an increase in messenger RNA (mRNA) expression between PNL (obtained during the third trimester) and TNL or TL. We chose chemokine receptors already associated with chemotaxis (Table[28−32]). In Figure 4, A, the mRNA abundance for receptors *CCR1*, *CCR3*, *CCR4*, *CCR5*, and *CXCR5* showed an increase between PNL and TL, whereas *CCR7* increased between PNL and TNL ($P<.05$). We next compared the expression levels of chemokine receptors by leukocytes with their migration numbers. Figure 4, B demonstrates chemokine receptor mRNA abundance correlated with migration. Five of the 6 chemokine receptors tested increased expression significantly ($P<.05$) in association with migration; only *CCR4* mRNA abundance was negatively correlated with migration to TL hFM chemoattractants.

Chemokines and cytokines increase in maternal serum with term labor onset

We examined maternal TNL and TL serum for changes in chemokine and cytokine concentrations using multiplex analysis and concluded that the following cytokines and chemokines significantly ($P<.05$) increase their concentration in maternal serum with term labor onset: CCL (C-C motif chemokine ligand) 11, CCL20, CCL21, CCL23, IFN (interferon)-γ, IL (interleukin)-4, IL-6, and IL-8 ($P<.05$) (Figure 5).

Term-labor human fetal membrane explant-conditioned medium both attracts and activates term-not-in-labor leukocytes

Conditioned media were collected from TNL and TL explants after a 24 hour incubation and were tested for chemoattractant activity. We found that the conditioned media from TNL and TL had chemoattractive activity, as they both increased the migration of TL leukocytes relative to the control medium ($P<.01$) (Figure 6, A).

We also tested whether the conditioned media from TNL or TL hFM explants could activate TNL leukocytes for migration. In this case, leukocyte responsiveness increased only after

A, mRNA expression of chemokine receptors on peripheral human leukocytes at PNL, TNL, and TL (*asterisk* denotes $P<.05$; *double asterisks* denote $P<.01$; n=3–6 for each time group). **B,** The relative mRNA abundance (X-axis) for the same chemokine receptors correlates with the migration of term leukocytes to term chemoattractants (Y-axis) in TL leukocytes (R=Pearson correlation test; n=12).

CCR, C-C motif chemokine receptor; *CXCR5*, C-X-C motif chemokine receptor 5; *mRNA*, messenger RNA; *PNL*, preterm not in labor; *TL*, term labor; *TNL*, term not in labor.

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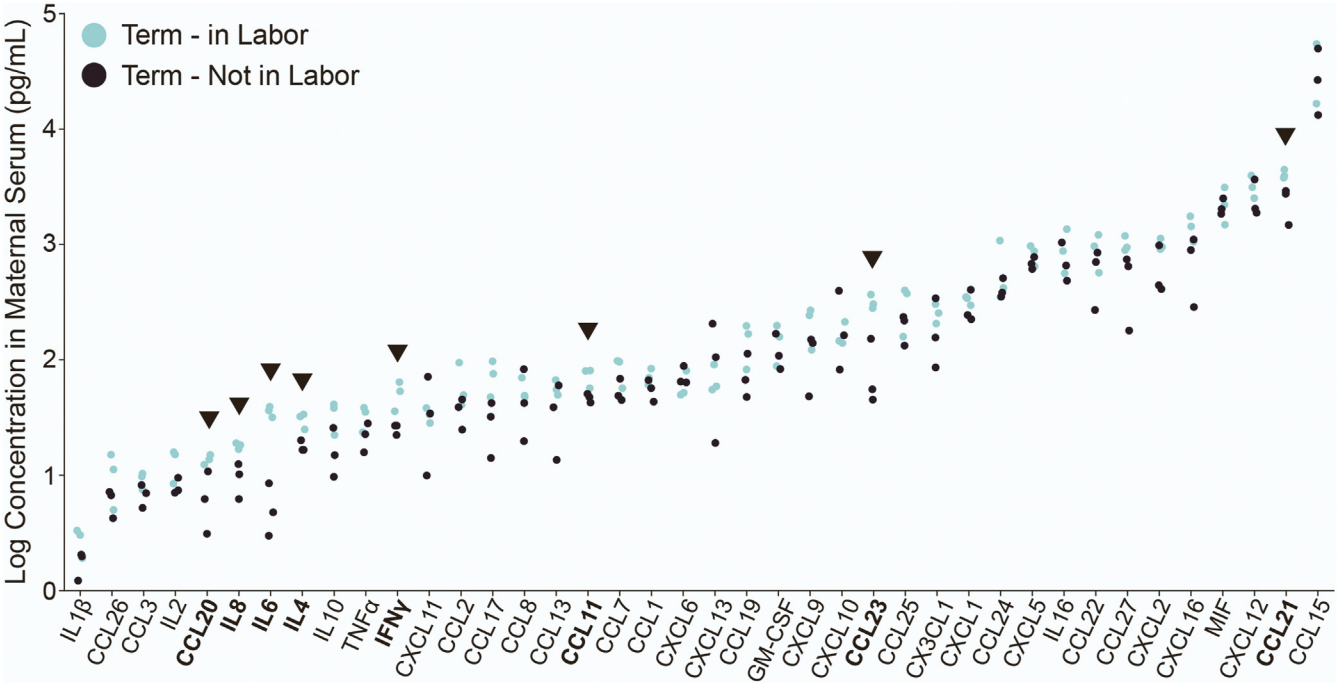
FIGURE 5
Comparison of 40 cytokines/chemokines from term not in labor and term in labor maternal serum



Serum was isolated from whole blood using centrifugation, and the serum components were quantified using multiplex screening analysis. Statistically significant differences are indicated by (*bold arrowheads*) ($P<.05$).

CCL, C-C motif chemokine ligand; *CXCL*, C-X-C motif chemokine ligand; *CX3CL1*, C-X3-C motif chemokine ligand 1; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *IL*, interleukin; MIF, macrophage migration inhibitory factor; *TNFα*, tumor necrosis factor α.

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exposure to the TL conditioned media ($P<.05$) (Figure 6, B).

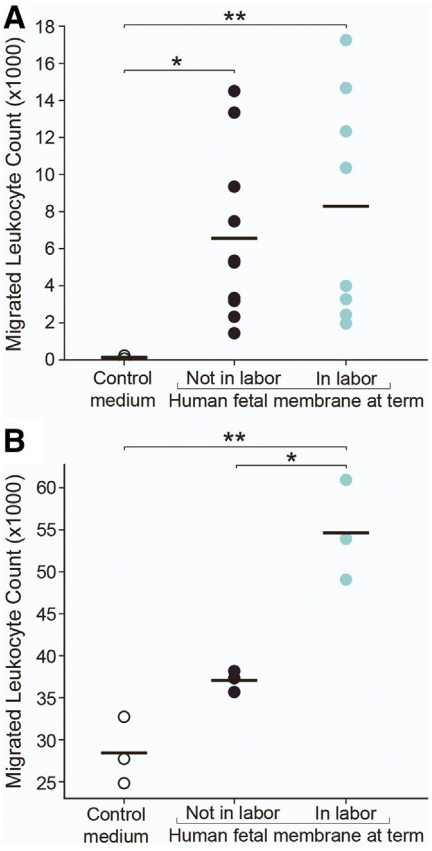
Maternal serum at term activates leukocytes for migration

Assuming that the fetal membrane/decidual chemoattractants diffuse into the maternal serum to activate circulating leukocytes for migration, we then tested whether late-gestation maternal serum could increase leukocyte migration to term chemoattractants (Figure 7). Leukocytes obtained from TNL women were exposed to serum isolated from either TNL or TL women for 1 hour and then tested for migration. Regardless of the serum type, leukocytes exposed to serum demonstrated increased migration to the term chemoattractive extracts. These data suggest that maternal serum contains factors that increase leukocyte responsiveness to hFM chemoattractants, and we suggest that the source of these factors is the hFM.

Leukocyte migration assay predicts delivery with high performance characteristics

The performance of the LMA is described in Figure 8. The cutoff for predicting delivery within 7 days at 4575 cells (Figure 8, A) was obtained by inputting the data for prediction within various days and using the Youden J index to calculate the best sensitivity and specificity pair.[25,26] The area under the curve was 82.4% using receiver-operating characteristic curve analysis (Figure 8, B). The cohort prevalence was 71.7%, and the calculated assay parameters are positive predictive value (96.1%; 49/51), negative predictive value (58.5%; 24/41), sensitivity (74.2%; 49/66), specificity (92.3%; 24/26), positive likelihood ratio (9.25; 0.74/0.08), and negative likelihood ratio (0.28; 0.26/0.92). It is useful to know the degree of confidence that a single LMA determination has to avoid unnecessary retesting. This can be estimated by replotting the migration data using a cost-curve plot analysis. Originally developed for economic analyses, the X-axis (probability cost) on the cost-curve represents the positive importance, which increases with the probability of the positive class, and the Y-axis represents the normalized expected cost or error rate when the misclassification costs are equal. The plot demonstrates that as the probability of the positive class increases, the error rate decreases. Applying this to our data indicates that the highest normalized "cost" (or error rate) is only 20% (Y-axis) (Figure 8, C).[27] This method indicates that there are few trivial classifiers of the analysis (Figure 8, C, dotted yellow-black line) in the practical operating range of the assay from approximately X=0.3 until approximately X=0.8 (Figure 8, C, black line). The straight-line nature of the curve in this range signifies that there is a single threshold value, meaning that 1 LMA determination produces results with a high degree of confidence.

FIGURE 6
hFM explants secrete chemotactic factors that activate leukocytes to respond to it



A, Conditioned medium from TNL (black) and TL (aquamarine) hFM explants attracted TL leukocytes in the LMA (*asterisk* denotes $P<.05$; n=8–9 for each treatment). **B,** Leukocytes obtained from TNL women were exposed to conditioned medium from TNL (black) and TL (aquamarine) explants for 1 hour and then tested for migration in the LMA using TL hFM homogenate (*asterisk* denotes $P<.05$; *double asterisks* denote $P<.01$; n=3 for each treatment).

hFM, human fetal membrane; *LMA*, leukocyte migration assay; *TL*, term labor; *TNL*, term not in labor.

Lee. Leukocyte migration before spontaneous term labor. Am J Obstet Gynecol 2024.

Comment

Principal findings

We used our published LMA to examine the physiology and timing of leukocyte migration in humans. Peripheral leukocytes isolated from women demonstrated peak migration-responsiveness during early labor with a rapid descent postpartum after the expulsion of the products of conception. Following leukocyte migration rates over the final weeks of pregnancy, we observed a trend of increased migration 2 weeks before delivery, with the most significant increase occurring in the last week before delivery (P=.012).

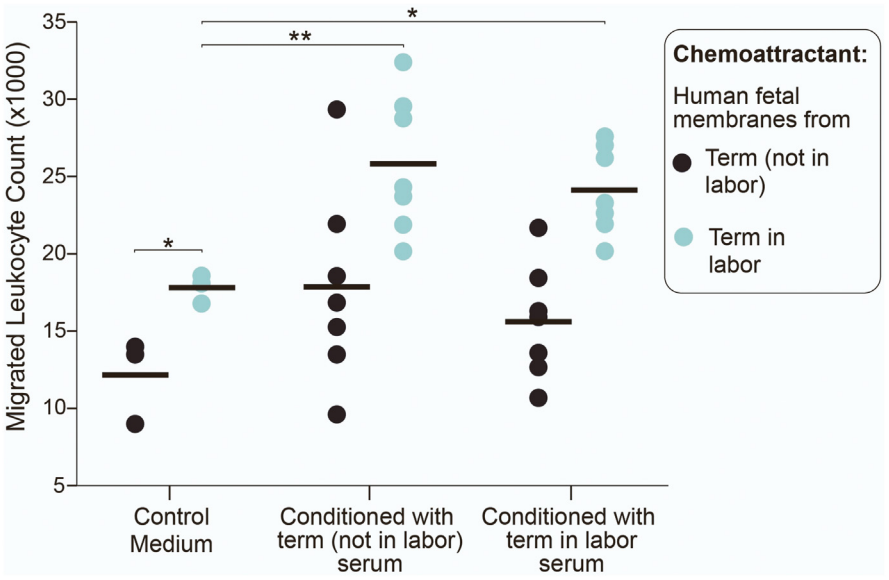
We hypothesize that this increase in migration before delivery is due to the release of more uterine chemoattractants as labor approaches. They enter maternal blood and prime or condition the leukocytes for migration. Our hypothesis was supported by an increase in the concentrations of 8 different chemokines and cytokines in maternal serum with labor onset (*CCL11, CCL20, CCL21, CCL23, IL4, IL6, IL8, IFNγ*) and an increase in the leukocyte mRNA abundance of several chemokine receptor genes (*CCR1, CCR3, CCR4, CCR5, CCR7, CXCR5*) that also correlated with increased migration in the LMA (all but CCR4). We replicated leukocyte priming ex vivo by exposing maternal peripheral leukocytes to conditioned media from term hFM explants and term maternal serum. The migration-responsiveness of these primed leukocytes increased in our LMA. Finally, we demonstrated that the LMA is sensitive, precise, and capable of predicting term delivery within 7 days with high validity for a single determination.

Results in the context of what is known

Nearly all species of leukocytes invade the uterus sometime during pregnancy.[1] Granulocytes, particularly neutrophils, as well as monocytes in peripheral blood, increase in number during late pregnancy.[16,33–35] They show functional changes that are relevant for pregnancy, including increasing numbers due to stress and endocrine and inflammatory stress mediators[36,37]; decreasing their rate of apoptosis[38]; and modulating macrophage phenotypes by suppressing their proinflammatory cytokine release.[39–42] We demonstrated that up to 2 weeks before delivery, leukocytes increase their responsiveness and migration to chemoattractants. This behavior serves as a proxy for uterine invasion.

The data and literature suggest that the fetal membranes are an important source of these mediators and that they have increasing potency with labor onset, which we confirmed using fetal membrane extracts and explants and maternal serum.[23,43] Intriguing questions include in which combinations and in which amounts the chemokines from the fetal membranes comprise the chemoattractants. For many years, Van Damme and his colleagues studied neutrophil chemoattraction to various chemokines and learned that in combination and varying concentrations, they were far better chemoattractants than they were individually because of synergistic effects.[44,45] We too suspect that chemokines from the fetal membranes in late gestation increase and combine to promote leukocyte invasion of the uterus for parturition.

FIGURE 7
Maternal serum activates leukocytes to respond to TL hFM chemoattractants



Leukocytes obtained from TNL women were exposed to serum collected from TNL and TL women for 1 hour (X-axis) and then tested for migration to either TNL (black) or TL (aquamarine) hFM chemoattractants using the leukocyte migration assay. TNL leukocytes conditioned in either TNL or TL serums demonstrated significant increases in migration to TNL and TL chemoattractants. *Asterisk* denotes $P<.05$; *double asterisks* denote $P<.01$ (n=3–7 for each treatment).

hFM, human fetal membrane; *TL*, term labor; *TNL*, term not in labor.

Lee. Leukocyte migration before spontaneous term labor. Am J Obstet Gynecol 2024.

Clinical implications

Women and their care providers value reliable information about the precise delivery timing. Our data show a 3-fold increase in leukocyte migration in women before term labor, which is valuable information that could be used to determine when to transport women who live in rural and remote locations to a tertiary center for delivery. The LMA may also be used to identify an inflammatory condition that increases leukocyte migration, leading to PTB.[14] More development of the assay is required before it will be practical for clinic or hospital use. Nevertheless, it has considerable potential for clinical applications. The physiological knowledge obtained in this study contributes to our understanding of human parturition.

Research implications

Many questions about these chemoattractants derive from this work, such as their chemical nature, how they activate neutrophils to become more responsive, and whether their production and secretion by the fetal membranes is stimulated by a factor released by the fetus before the onset of labor.

Strengths and limitations

It appears that the LMA is a useful bioassay for studying leukocyte migration behavior in relation to term delivery. We also demonstrated in several ways that the chemoattractants released by the fetal membranes (probably into maternal blood) are most likely the stimulus for enhanced leukocyte migration at term. An intriguing question for future investigation will be the role of the chemoattractants in initiating labor.[46–51] One limitation is that considerably more work is required to ascertain the chemical nature of these chemoattractants and the proportions of various constituents.[41,42] Another limitation is that although the LMA shows promise as a diagnostic tool using retrospective cohorts, considerably more development is required before it is ready for use in a clinical laboratory and approved as a diagnostic tool.

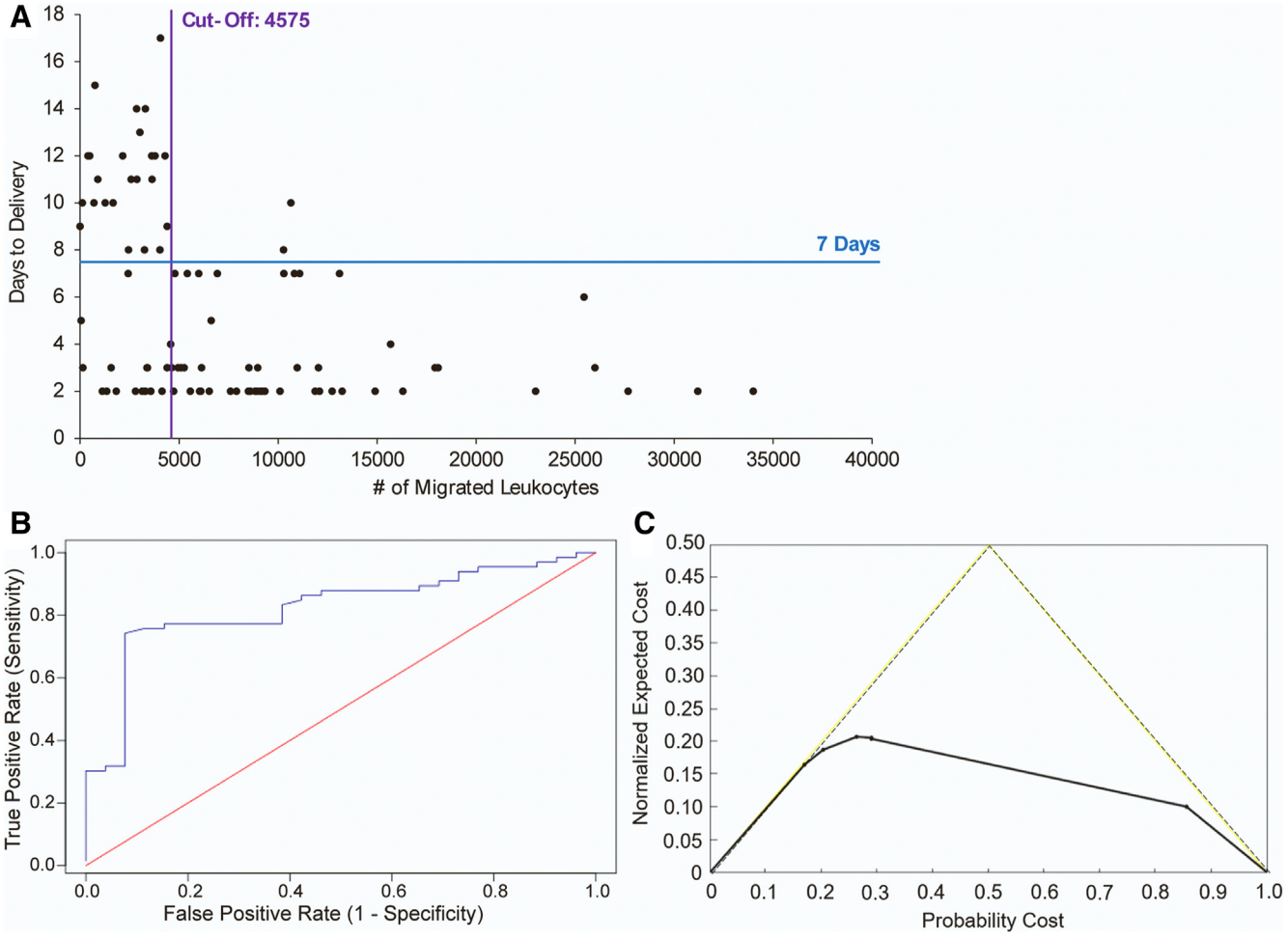
Conclusions

Our data support the hypothesis that chemoattractants released by hFMs at term are critically important regulators of leukocyte activation and migration, and this process starts at least 1 week before delivery. We also showed that our patented LMA can predict labor within 7 days, thus demonstrating potential as a labor timing diagnostic tool. Given that hFM chemoattractants also play a crucial role in the LMA, determining their composition is of clinical importance. ■

GLOSSARY

ANOVA Analysis of variance
RM-ANOVA Repeated measures ANOVA
AUC Area under the curve
CCL C-C motif chemokine ligand
CCR C-C motif chemokine receptor
CXCL C-X-C motif chemokine ligand
CXCR5 C-X-C motif chemokine receptor type 5
CX3CL1 C-X3-C motif chemokine ligand 1
DMEM Dulbecco's Modified Eagle Medium
FSC Forward scatter in flow cytometry
GM-CSF Granulocyte-macrophage colony-stimulating factor
hFM Human fetal membranes
IFN-γ Interferon-γ
IL Interleukin
LMA Leukocyte migration assay
MIF Macrophage migration inhibitory factor
mRNA Messenger RNA
P Probability level that the null hypothesis is false
PBS Phosphate-buffered saline
PNL Preterm not in labor (normal pregnancy before term)
PTB Preterm birth
RPMI Roswell Park Memorial Institute 1640 medium
SSC Side scatter in flow cytometry
TL Term in labor
TNFα Tumor necrosis factor α
TNL Term not in labor

FIGURE 8
Performance of the leukocyte migration assay



A, The migration patterns of the assay produced distinct cutoff values for participants who would deliver within 7 days at 4575 total migrated cells in Chongqing. **B,** The receiver-operating characteristic curve is shown with an area under the curve of 83%. **C,** The cost-curve plot of the migration data demonstrates that the probability cost function (X-axis) produces the highest normalized expected cost or error rate of 20% (Y-axis) when the probability cost is low (approximately 30%), and the lowest error rate (10%) when the probability cost is highest at 80%. These parameters define the operating range of the probability cost given that the curve diverts from the distribution of the yellow and black-dotted line that describes trivial classifiers.

Lee. Leukocyte migration before spontaneous term labor. Am J Obstet Gynecol 2024.

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