# UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
## POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE MEDICINA

BIOLOGÍA EXPERIMENTAL

# CARACTERIZACIÓN CELULAR Y FUNCIONAL DEL MICROAMBIENTE CORIODECIDUAL DE LAS MEMBRANAS FETALES AL TÉRMINO DE LA GESTACIÓN HUMANA

# T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE

**DOCTORA EN CIENCIAS**

P R E S E N T A

MARISOL CASTILLO CASTREJÓN

**TUTOR PRINCIPAL DE TESIS:** DR. FELIPE VADILLO ORTEGA
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MÉXICO D.F., JUNIO 2014

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UNAM
POSGRADO
Ciencias Biológicas

COORDINACIÓN

**Dr. Isidro Ávila Martínez**
**Director General de Administración Escolar, UNAM**
**P r e s e n t e**

Me permito informar a usted que el Subcomité de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, en su sesión ordinaria del día 03 de junio de 2013, aprobó el jurado para la presentación de su examen para obtener el de grado de **DOCTORA EN CIENCIAS** de la alumna **CASTILLO CASTREJÓN MARISOL** con número de cuenta **506021343** con la tesis titulada **"CARACTERIZACIÓN CELULAR Y FUNCIONAL DEL MICROAMBIENTE CORIODECIDUAL DE LAS MEMBRANAS FETALES AL TÉRMINO DE LA GESTACIÓN HUMANA"**, realizada bajo la dirección del **DR. FELIPE VADILLO ORTEGA:**

| | |
|---|---|
| Presidente: | DRA. YOLANDA LÓPEZ VIDAL |
| Vocal: | DR. SAMUEL CANIZALES QUINTEROS |
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| Suplente: | DRA. GLORIA SOLDEVILA MELGAREJO |
| Suplente: | DR. EDGAR ARTURO ZENTENO GALINDO |

Sin otro particular, me es grato enviarle un cordial saludo.

**A T E N T A M E N T E**
**"POR MI RAZA HABLARÁ EL ESPÍRITU"**
**Cd. Universitaria, D.F., a 20 de mayo de 2014**

**DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA**
**COORDINADORA DEL PROGRAMA**

# AGRADECIMIENTOS

Al Posgrado en Ciencias Biológicas, UNAM; por permitirme continuar mi formación académica, y de esta manera permitir contribuir al crecimiento científico de me país.

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**A mi Familia, por todo su apoyo, enseñanzas**
**y su amor incondicional.**
**G R A C I A S**

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# ABREVIATURAS

| | |
|---|---|
| MCA | Membrana corioamniótica humana |
| RPM | Ruptura prematura de membranas |
| RPMP | Ruptura prematura de membranas pretérmino |
| MMPs | Metaloproteasas de matriz extracelular |
| MT-MMPs | Metaloproteasas de matriz extracelular de tipo membrana |
| TIMPs | Inhibidores tisulares específicos de metaloproteasas de matriz extracelular |
| NGAL | Gelatinasa B de neutrófilos asociada a lipocalina |
| SDG | Semanas de gestación |
| PBS | Amortiguador salino de fosfatos |
| PAGE | Electroforesis en geles de poliacrilamida |
| SDS | Dodecil sulfato de sodio |
| HLA | Hidrolizado de lactoalbúmina |
| IL | Interleucina |
| TNF-$\alpha$ | Factor de necrosis tumoral alfa |
| MCP-1 (CCL2) | Proteina quimioatrayente de monocitos-1 |
| MIP-1$\alpha$ (CCL3) | Proteína inflamatoria de macrófagos |
| MIP-1$\beta$ (CCL4 | Proteína inflamatoria de macrófagos |
| NKs | Células asesinas naturales |
| IFN-$\gamma$ | Interferón gama |
| sIL-1Ra | Antagonista del receptor de IL-1 |

# ÍNDICE DE FIGURAS Y TABLAS

# RESUMEN

**Antecedentes:** El espacio coriodecidual es un microambiente que se enriquece en mediadores del trabajo de parto al final de la gestación humana. El incremento en la concentración local de quimiocinas, citocinas, prostaglandinas y metaloproteasas de matriz extracelular se ha vinculado con la activación de eventos del trabajo de parto. Sin embargo, se desconocen las fuentes celulares de estos compuestos que participan en el proceso inflamatorio asociado al trabajo de parto.

**Objetivo:** Analizar la capacidad de secreción *in vitro* de mediadores de la inducción del trabajo de parto por leucocitos aislados del espacio coriodecidual de embarazos humanos a término y compararlos con poblaciones equivalentes de la circulación placentaria.

**Material y métodos:** Se desarrolló un método para aislar leucocitos del espacio coriodecidual de membranas corioamnióticas obtenidas de embarazos de término y leucocitos de la circulación placentaria. Las subpoblaciones de leucocitos de ambos compartimentos fueron fenotipificadas por citometría de flujo utilizando anticuerpos monoclonales conjugados a un fluorocromo. Se analizaron 11 citocinas (IL-1$\alpha$, IL-1$\beta$, IFN-$\gamma$, TNF-$\alpha$, IL-6, IL-17, IL-2, IL-1ra, IL-10, IL-4 y sIL-1Ra), 4 quimiocinas (MIP-1$\alpha$, MIP-1$\beta$, IP-10, MCP-1 e IL-8) y 3 metaloproteinasas (MMP-9, MMP-2 y MMP-3) relacionadas con el evento inflamatorio.

**Resultados:** Los leucocitos aislados de la coriodecidua fueron caracterizados en su mayoría como linfocitos T, monocitos y células asesinas naturales. Los leucocitos aislados de coriodecidua secretan un patrón pro-inflamatorio caracterizado por IL-6 y TNF-$\alpha$; secretan quimiocinas tales como CCL2 y CCL3 de manera diferencial con los aislados de la circulación placentaria. Los leucocitos coriodeciduales secretan y activan a la MMP-9 en comparación los leucocitos placentarios que no secretan ni activan esta enzima. Se demostró la presencia de MMP-3 que coincide con la aparición de las formas activas de MMP-9.

**Conclusiones:** Los leucocitos presentes en el entorno intrauterino están enriquecidos en linfocitos T hacia el final de la gestación. Estas células exhiben propiedades funcionales características como la secreción y activación de la MMP-9 y la secreción de mediadores que constituyen una red de señalización compleja capaz de inducir cambios simultáneos en el miometrio, en las membranas corioamnióticas y en el cérvix. Estos datos permiten postular que existen mecanismos de reclutamiento de subpoblaciones de leucocitos específicas para desencadenar el trabajo de parto humano a término.

# ABSTRACT

**Background:** The choriodecidual microenvironment is enriched in mediators of labour at the end of human gestation. The increase in the local concentration of chemokines, cytokines, prostaglandins and extracellular matrix metalloproteinases has been linked to the activation of the events associated to normal human labour. However, the cellular sources of these compounds involved during labor are not completely elucidated.

**Aims:** To analyze the capacity of *in vitro* secretion of labor-induced mediators by choriodecidual space leukocytes isolated from term human fetal membranes and compared them with equivalent cell populations in placental blood circulation.

**Method of study:** A protocol was developed to isolate leukocytes from choriodecidual space from term fetal membranes and from placental blood circulation. Leukocyte subpopulations were characterized from both compartments by flow cytometry using specific fluorochrome-conjugated antibodies. Leukocyte-enriched preparations were maintained in culture and secretion of 11 cytokines (IL-1α, IL-1ß, IFN-δ, TNF-α, IL-6, IL-17, IL-2, IL-1ra, IL-10, IL-4 and sIL-1Ra), 4 chemokines (MIP-1α, MIP-1ß, IP-10, MCP-1 and IL-8) and 3 metalloproteinases (MMP-9, MMP-2 and MMP-3) related to the inflammatory event were evaluated.

**Results:** The leukocytes isolated from the coriodecidua were represented by T lymphocytes, monocytes and natural killer cells. Leukocytes isolated from coriodecidua secrete a pro-inflammatory pattern characterized by IL-6 and TNF-α and a chemokine pattern of CCL2 and CCL3 differentially from placental blood leukocytes. The coriodeciduales leukocyte secrete and activate MMP-9. Placental blood leukocytes do not secrete or activate this enzyme. The presence of MMP-3, which coincides, with the appearance of the active forms of MMP-9

**Conclusions:** Choriodecidual space by the end of gestation is enriched with leukocytes represented mainly by T cells and monocytes/macrophages. Choriodecidual leukocytes exhibit specific characteristics such as secretion and activation of MMP-9, and the secretion of mediators, which are a complex network of signaling to induce simultaneous changes in the myometrium, fetal membranes and cervix. We postulate the existence of a mechanism to allow specific leukocytes recruitment into fetal and maternal tissues that may be linked to the induction and progression of human labor.

# PREFACIO

El nacimiento pretérmino es la primera causa de muerte neonatal en países en vías de desarrollo y contribuye de forma significativa a la tasa de mortalidad infantil, responsable de 3.1 millones de muertes al año a nivel mundial. Nacer antes del término incrementa en un 50% el riesgo de muerte neonatal por causas como infecciones y neumonía (Liu L et al., 2000). El costo de atención de los neonatos pretérmino es elevado y requiere de atención hospitalaria de alta especialidad (Thompson et al., 2006), además los sobrevivientes pueden tener secuelas que van desde sordera o debilidad visual hasta discapacidad grave por daño neurológico y mayor predisposición a enfermedades crónico-degenerativas en la etapa adulta (Murray et al., 2012).

La Organización Mundial de la Salud define al nacimiento pretérmino como el nacimiento antes de completar la semana de gestación 37 (259 días) a partir del último periodo de menstruación de una mujer (WHO., 1976). Se considera un síndrome y es un proceso multifactorial donde interaccionan varios factores que dan como resultado la pérdida de la quiescencia y genera la activación del útero que sucede antes del término de la gestación. Los precursores del nacimiento pueden variar desde factores maternos, sociales y ambientales. A pesar de muchos años de investigación, las causas del nacimiento pretérmino no han sido definidas y por ello, no existen medidas preventivas para su desarrollo. Por esta razón, es una prioridad desarrollar mayor conocimiento que sea de utilidad para el diagnóstico y/o tratamiento de las patologías asociadas al nacimiento pretérmino.

La patología que se asocia de forma más común al nacimiento pretérmino es la ruptura prematura de membranas, que explica casi el 50% de estos casos. Los mecanismos celulares y moleculares que llevan a la ruptura fisiológica de las membranas no han sido elucidados en su totalidad. Este estudio pretende ampliar el conocimiento sobre los mecanismos implicados en la ruptura de las membranas corioamnióticas durante el trabajo de parto normal, lo que podría dar pauta para esclarecer la forma y el tiempo en que el microambiente uterino dicta el término de la gestación en el ser humano. Conociendo el proceso que lleva a la ruptura de las membranas corioamnióticas en condiciones normales, podremos esclarecer los mecanismos que se activan de manera asincrónica y que condicionan el nacimiento pretérmino.

# INTRODUCCIÓN

## Trabajo de parto humano

Durante casi todo el embarazo humano el útero se mantiene relativamente sin actividad contráctil, el cérvix permanece rígido y cerrado y las membranas corioamnióticas se mantienen estructuralmente intactas. Al término de la gestación cuando el feto está totalmente desarrollado el útero se contrae, el cérvix se dilata y las membranas corioamnióticas se rompen, dando como resultado el nacimiento. A este proceso fisiológico se le denomina trabajo de parto y se logra por una secuencia de eventos coordinados por una red de señalización autocrina/paracrina que modifican el ambiente uterino donde intervienen mensajes maternos y fetales (Bryant-Greenwood *et al.*, 2000; Garfield *et al*. 2007).

Los mecanismos celulares que inician el trabajo de parto al final de la gestación han sido parcialmente descritos ya que los modelos animales proporcionan información limitada. Aunque se desconoce la totalidad de los detalles del inicio y regulación del proceso del trabajo de parto normal, en los últimos años se han comprendido mecanismos moleculares y celulares que están implicados en el nacimiento humano.

El trabajo de parto tiene dos fases, una subclínica que se inicia semanas antes de la aparición de signos y los síntomas propios del trabajo de parto y que constituyen la segunda fase. La fase subclínica consiste en cambios en el miometrio que provocan el aumento de las conexiones intercelulares y su excitabilidad lo que prepara al útero para contraerse. Al mismo tiempo, en el cérvix y en las membranas corioamnióticas se inicia el arribo de diferentes subpoblaciones de leucocitos cuya función ha sido poco caracterizada. La fase clínica inicia cuando las contracciones uterinas son intensas y rítmicas. Cuando éstas últimas son efectivas, el cérvix inicia su maduración que se define por dilatación y borramiento. El último evento del trabajo de parto es la ruptura de las membranas corioamnióticas, lo que libera el líquido amniótico y deja libre al producto para su expulsión cuando las contracciones uterinas alcanzan su pico de eficiencia.

La parte menos comprendida de estos procesos es la red de señalización que coordina todos estos eventos y aunque conocemos desde hace tiempo diferentes compuestos

uterotónicos, como las prostaglandinas y la oxitocina, así como compuestos que inducen la maduración cervical y la ruptura de las membranas corioamnióticas, no se ha descrito un modelo de señalización que orqueste estos fenómenos.

Es de nuestro interés el estudio del mecanismo de ruptura de las membranas corioamnióticas durante el trabajo de parto normal. Por ello se describe a continuación la estructura y las funciones de las membranas corioamnióticas durante la gestación humana.

## Las membranas corioamnióticas

A lo largo del embarazo, el embrión humano desarrolla tejidos extraembrionarios accesorios que incluyen a la placenta y a las membranas corioamnióticas. En conjunto proveen al producto de diferentes mecanismos de intercambio, protección y control de las relaciones con el ambiente extrauterino.

Se han descrito diferentes funciones de las membranas corioamnióticas (Bryant-Greenwood *et al*. 1998; Zaga-Clavellina *et al.*, 2004):

- Contener y proteger al feto durante la gestación
- Regular el contenido y composición del líquido amniótico
- Formar una barrera inmunológica
- Participar activamente durante el trabajo de parto como fuente de mediadores con actividad biológica y responder a diferentes señales provenientes tanto del producto como de la madre

El embarazo humano requiere que las membranas corioamnióticas sean lo suficientemente fuertes y elásticas para resistir el peso y los movimientos fetales vigorosos principalmente al término de la gestación. Su arreglo y composición específica hace que esto sea posible. Las membranas corioamnióticas son tejidos multilaminares integrados por dos principales capas: el amnios que está en contacto con el líquido amniótico y el corion que está en contacto con la decidua materna *(figura1)*(Bryant-Greenwood *et al.,* 2000). La coriodecidua es el área de contacto entre los tejidos maternos y fetales, ésta incluye leucocitos infiltrados, células deciduales y trofoblastos embebidos en matriz extracelular.

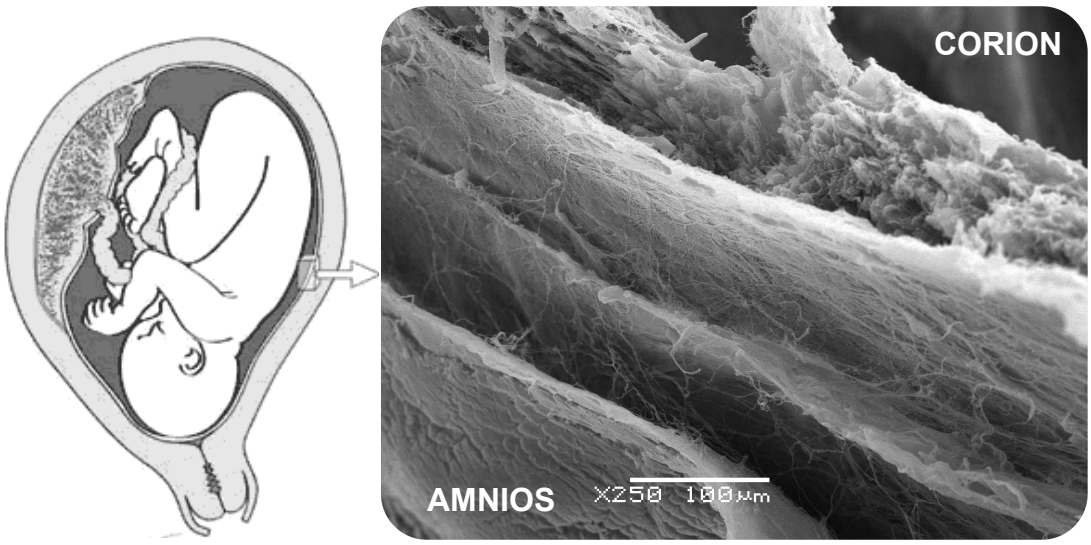


**Figura 1. Representación esquemática de la localización de las membranas fetales humanas.** Micrografía electrónica de barrido. Membranas corioamnióticas: corion y amnios. Aumento x250. Fotografía: Marisol Castillo Castrejon.

Las membranas corioamnióticas están constituidas por células epiteliales, mesenquimales y citotrofoblásticas, embebidas en una matriz extracelular rica en agua y colágena. Los componentes de la matriz extracelular son responsables de las propiedades biomecánicas del tejido y son elementos de señalización que permiten responder a las necesidades cambiantes del tejido. Los principales componentes de la matriz extracelular de las membranas corioamnióticas son: la colágena, la elastina, la laminina y la fibronectina. Los tipos de colágena más abundantes en esta matriz son: I, III, IV, V y VI. Estos tejidos son únicos en su composición tisular; de manera característica contiene mayor proporción de tejido conectivo en comparación de su masa celular. En este sentido, el amnios solamente consiste en un capa de células epiteliales que se asienta en una membrana basal, que a su vez se encuentra anclada a una capa de tejido conectivo cuya estructura única resulta en una red tridimensional densa y resistente, que es responsable de al menos el 75% de la fuerza tensil del tejido y se conoce como capa compacta. La composición de este sustrato se basa en una arreglo específico de fibras de colágenas tipo I y III, que son estabilizadas por una red de colágenas tipo IV, V y VI (Vadillo-Ortega *et al.*, 2005).

## Ruptura de las membranas corioaminóticas

La evolución de la comprensión de la ruptura de las membranas corioamnióticas ha permitido integrar un esbozo del posible mecanismo implicado que comprende lo siguiente:
La primera hipótesis, propone que la ruptura de las membranas corioamnióticas se debe factores físicos como la presión intrauterina producto de las contracciones uterinas. Durante los años noventa, se logró identificar un mecanismo de daño enzimático en la ruptura de estos tejidos. La evidencia mostró que la ruptura está relacionada a cambios morfológicos provocados por la degradación del tejido conectivo tanto del amnios como del corion (Parry S *et al.*, 1998). La degradación selectiva del tejido conectivo está mediada principalmente por una familia de endopeptidasas denominadas metaloproteasas de matriz extracelular (MMPs).

Hasta la fecha, el mecanismo central de daño que causa la ruptura fisiológica de las membranas corioamnióticas involucra la inducción, la expresión, la secreción y la activación de las MMPs por células infiltradas y locales que se relaciona con la temporalidad de los otros eventos del trabajo de parto. De manera que lleva a la pérdida de la estructura, a la disminución de su resistencia mecánica y hasta el punto en que el contenido intrauterino exceda su capacidad ténsil y aparezca la ruptura tanto en condiciones fisiológicas como patológicas (Vadillo Ortega *et al.* 1995). Sin embargo, aún se encuentra en estudio la secuencia de eventos que terminan en la inducción enzimática que conlleva a la ruptura de las membranas (Uchide *et al.*2000).

## Metaloproteasas de matriz extracelular

La degradación del tejido conectivo de las membranas corioamnióticas está mediado por las MMPs, una familia de enzimas dependientes de zinc y calcio, que tienen afinidad por diferentes sustratos de la matriz extracelular (Nagase *et al.*, 1999). Esta familia de enzimas se caracteriza por la presencia de dominios proteicos conservados: péptido señal cuya función es dirigir a la proteína hacia el lumen del retículo endoplásmico para ser exportada por la célula; el propéptido, de aproximadamente 100 residuos que puede ser hidrolizado durante el proceso de activación; el dominio catalítico que contiene el sitio activo que permite la unión a un ión de $Zn^{2+}$ a través de tres residuos de histidina y una molécula de agua (Woessner *et al.* 2000).

Las MMPs pueden variar por su peso molecular, estructura, afinidad a diferentes sustratos y por los mecanismos de regulación (Visse et al., 2003). Al menos 20 miembros de la familia de las MMPs han sido reportados y por su afinidad a diferentes sustratos se clasifican en: *1.* colagenasas intersticiales (MMP-1,-8,-13 y -18); *2.* gelatinasas A y B (MMP-2 y -9); *3.* estromelisinas (MMP-3, -10 y -11); *4.* matrilisina o MMP-7; y *5.* metaloproteasas de matriz extracelular de tipo membrana (MT-MMPs) (MMP-14, -15, -16 y -17) (Overall et al., 2002). Son sintetizadas y secretadas por diferentes tipos celulares tales como los fibroblastos, el endotelio, las células epiteliales y las células inmunes como los macrófagos, los eosinófilos, los neutrófilos y los mastocitos (Sternlicht et al., 2001)

Ha sido posible identificar que en asociación al desarrollo del trabajo de parto normal y en la ruptura de las membranas corioamnióticas se expresan al menos cinco diferentes miembros de la familia de las MMPs, que incluyen a la MMP-1, MMP-2, MMP-3, MMP-7 y principalmente MMP-9 (Labrie et al., 2013). Cada una de ellas muestra diferente afinidad por algún componente de la matriz extracelular y contribuyen a la degradación e la capa compacta y de otros estratos de las membranas corioamnióticas (Fortunato et al., 2002). Todas estas enzimas son secretadas por las células locales y de la coriodecidua en los días previos a la ruptura de las membranas corioamnióticas. Son secretadas al espacio extracelular en forma de zimógenos y por ello requieren de otras proteasas para su activación (Lee et al., 2004).

Se conoce poco sobre la activación fisiológica de las MMPs, pero existe evidencia de que las membranas corioamnióticas cuentan con un sistema que no ha sido descrito en otros tejidos y que le permite almacenar grandes cantidades de MMPs en forma latente en complejos multienzimáticos asociados a la matriz extracelular, que es desensamblado al momento del trabajo de parto y que permite la liberación de cantidades masivas de enzimas activas (Meraz-Cruz et al., 2006). Este sistema favorecerá de manera rápida la ruptura de las membranas corioamnióticas en sincronía con los eventos del trabajo de parto.

## El ambiente pro-inflamatorio en las membranas fetales

Los mecanismos que llevan al trabajo de parto al término de la gestación no han sido comprendidos en su totalidad. Sin embargo, el progreso del trabajo departo está mediado por cambios anatómicos, bioquímicos, endocrinológicos e inmunológicos que suceden en los diferentes tejidos maternos y fetales involucrados. La activación de los mecanismos asociados a la inflamación han sido propuestos como parte del inicio del trabajo de parto. Dicha activación está asociada al arribo de células del sistema inmunológico a los tejidos gestacionales (Young et al., 2002), tales como el cérvix (Bokstrom et al., 1997), el útero (Thompson et al., 1999) y las membranas fetales; que se caracteriza por la secreción de citocinas, quimiocinas y otros mediadores de la inflamación.

Se ha descrito que las diferentes subpoblaciones que arriban al espacio coriodecidual condicionan un microambiente inflamatorio que posiblemente este involucrado en la ruptura de las membranas fetales durante el trabajo de parto. Diferentes células de las membranas fetales pueden contribuir a la secreción de citocinas y MMPs. Sin embargo, el análisis del origen celular de algunas citocinas como el factor de necrosis tumoral alfa (TNF-α), la interleucina 1-beta (IL-1β) y la MMP-9 en tejidos gestacionales han demostrado que el infiltrado leucocitario también contribuye en la secreción de estas moléculas que coincide con el evento del trabajo de parto (Kelly RW., 1996). Estudios previos de nuestro grupo de trabajo han evidenciado el arribo de leucocitos a través de procesos quimiotácticos coordinados por las membranas fetales durante el trabajo de parto (Gomez-Lopez et al., 2009).

# ANTECEDENTES

Durante varios años el grupo de investigación al que pertenezco ha aportado algunas contribuciones sobre la participación del sistema inmunológico en los compartimentos fetales y maternos durante el trabajo de parto. Hemos demostrado que en las membranas fetales el trabajo de parto involucra procesos que emulan a la respuesta inflamatoria local que antecede y facilita su ruptura al término de la gestación.
En modelos experimentales, hemos aportado evidencia que las membranas corioamnióticas secretan diferentes señales quimiotácticas que atraen a diferentes subpoblaciones de leucocitos al espacio coriodecidual y que lo hacen en coordinación con el inicio de la fase subclínica del trabajo de parto (Gomez-Lopez et al., 2009). Las células atraídas por la membranas se enriquecen en la sangre del ambiente uterino y placentario e infiltran el espacio virtual que constituye la frontera entre los tejidos maternos y fetales, denominado coriodecidua.

Conforme a nuestra hipótesis, estas células inducen respuestas funcionales en las células residentes de las membranas corioamnióticas que se caracterizan por la secreción de compuestos con efectos sobre los diferentes tejidos que intervienen en el trabajo de parto. Esto incluye por lo menos, la secreción de compuestos uterotónicos como prostaglandinas y oxitocina, así como la secreción de mediadores del proceso inflamatorio y metaloproteasas de matriz extracelular. Los compuestos uterotónicos originados en las membranas corioamnióticas tendrían efecto sobre la musculatura uterina, las metaloproteasas condicionarían la degradación de las membranas corioamnióticas, las quimiocinas y citocinas pro inflamatorias favorecerán la activación del tejido. De esta manera se lograrían dos de las tres fases del trabajo de parto con la participación de éste sistema residente en las membranas corioamnióticas.

La caracterización inicial de los leucocitos que se enriquecen en el ambiente uterino y en la coriodecidua por técnicas histológicas, revelan la presencia de linfocitos T, monocitos y granulocitos. El dato más llamativo es la presencia de una subpoblación de linfocitos CD3+, MMP-9+ e IL-1$\beta$+, que no había sido descrita hasta ahora (Gomez-Lopez et al., 2011).

En trabajos previos de nuestro grupo y otros, se ha demostrado que la metaloproteinasa-9 (MMP-9) es un mediador de la degradación del tejido conectivo de las membranas fetales y responsable de su ruptura durante el trabajo de parto (Estrada-Gutierrez et al., 2005). Por otro lado, diferentes grupos hemos aportado información para señalar a la IL-1β y TNF-α como una señal primaria en la inducción de síntesis de diferentes moléculas efectoras, tales como prostaglandinas, oxitocina y MMPs.

Hallazgos previos nos permiten plantear un mecanismo para la ruptura de las membranas fetales al término de la gestación que se representa de manera esquemática *(figura 2)*. Una población de linfocitos que es reclutada en la interfase coriodecidual, aporta al menos una señal primaria pro-inflamatoria (TNF-α, IL-6 e IL-1β) que tiene efectos paracrinos sobre las diferentes subpoblaciones de la membrana corioamniótica. En respuesta a esta señal, las células deciduales, el trofoblasto, los fibroblastos y las células del epitelio amniótico, responden activando la expresión de diferentes proteínas, que incluyen enzimas como la prostaglandina sintetasa (COX-2), metaloproteasas como MMP-1, MMP-2, MMP-3, MMP-9, activadores de metaloproteasas como el activador de plasminógeno (uPA) y cantidades adicionales de citocinas pro-inflamatorias.

Todos estos compuestos se integran como la señal primaria de respuesta o mediadores primarios que a continuación ejercerán sus efectos en forma de cascada y las células locales responderán con la secreción de compuestos efectores como prostaglandinas, oxitocina y MMP-9. Las prostaglandinas y la oxitocina ejercerán efecto uterotónico y la MMP-9 será activada por las proteasas mencionadas arriba, para catalizar la degradación del tejido conectivo de las membranas corioamnióticas.

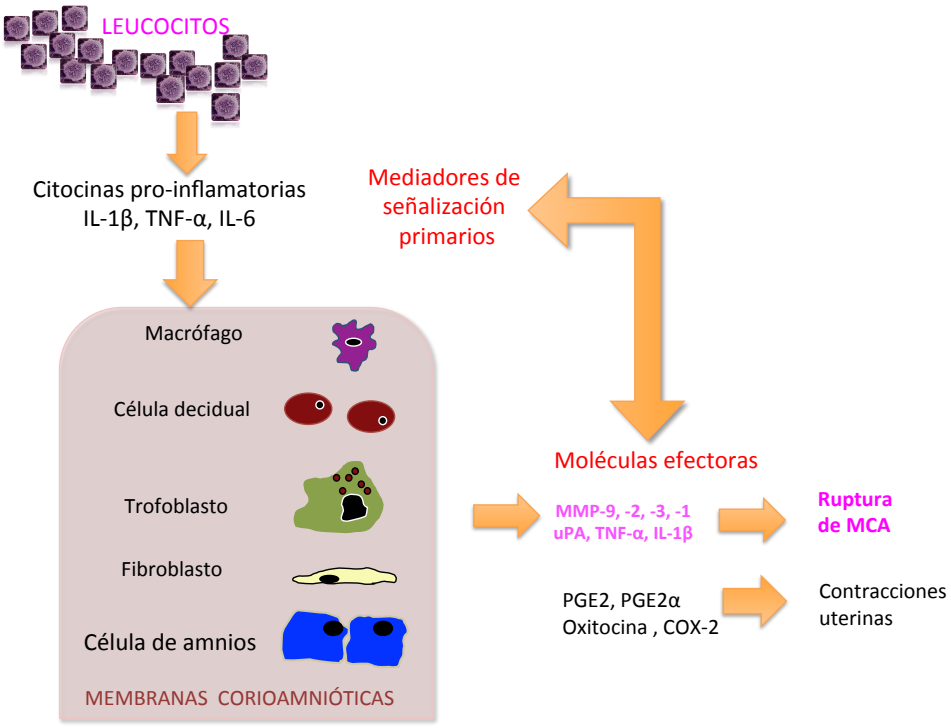


**Figura 2.** Modelo propuesto de señalización durante el trabajo de parto al término de la gestación humana. MCA, membrana corioamniótica.

# JUSTIFICACIÓN

Este proyecto aborda el estudio de una parte del microambiente uterino que es posible que coordine la ruptura de las membranas corioamnióticas y que incluye el análisis de la red celular que participa, así como la temporalidad de la secreción de moléculas señalizadoras y efectoras que participan en la regulación de la degradación del tejido conectivo de las membranas fetales antes durante el trabajo de parto humano al término de la gestación.

# HIPÓTESIS

Las células que arriban al espacio coriodecidual participan en la secreción de mediadores asociados la degradación del tejido conectivo de las membranas corioamnióticas previo al trabajo de parto humano.

# OBJETIVO GENERAL

Investigar si las células que infiltran el espacio coriodecidual previo al trabajo de parto, secretan mediadores responsables de la ruptura de las membranas corioamnióticas al término de la gestación.

# OBJETIVOS ESPECÍFICOS

1. Caracterizar el fenotipo de las células que infiltran el espacio coriodecidual al término de la gestación humana y compararlos con el compartimiento placentario.
2. Evaluar *in vitro* la cinética de producción de mediadores primarios de señalización (citocinas pro-inflamatorias, anti-inflamatorias y quimiocinas) por las diferentes subpoblaciones de leucocitos provenientes del espacio coriodecidual y placentario al término de la gestación.
3. Evaluar *in vitro* la cinética de producción de la metaloproteasa de matriz extracelular 9 por los leucocitos coriodeciduales y placentarios al término de la gestación.

# MATERIAL Y MÉTODOS

A continuación se describe el modelo experimental que se utilizó para evaluar la participación de los leucocitos coriodeciduales en la secreción de mediadores primarios y moléculas efectoras asociados a la degradación de las membranas corioamnióticas al término de la gestación. Con el propósito de realizar una comparación en los perfiles de secreción de moléculas se utilizó a los leucocitos provenientes de la circulación placentaria.

## Muestras biológicas

Las membranas corioamnióticas y la sangre de la circulación placentaria que se utilizaron provinieron de pacientes con los siguientes criterios de inclusión:
Mujeres sometidas a cesárea por alguna indicación obstétrica o ginecológica (desproporción cefalopelvica, productos en presentación pélvica, situación transversa, periodo intergenésico corto, cérvix desfavorable, sufrimiento fetal agudo), de más de 37 semanas de gestación, embarazo con único producto, en las que se documentó ausencia de trabajo de parto (ausencia de contracciones uterinas efectivas, sin dilatación cervical y membranas fetales integras), sin evidencia de infección o alguna patología durante el embarazo. No se incluyó a un grupo de mujeres con embarazo a término, con trabajo de parto y cuya resolución fue por vía vaginal por las condiciones experimentales necesarias para la extracción y cultivo de leucocitos coriodeciduales.

Las pacientes fueron identificadas e invitadas a participar en la consulta de urgencias del Hospital Materno Infantil Inguarán de la Secretaría de Salud del D.F. Las mujeres brindaron su consentimiento de participación por escrito. Se utilizó como criterio de eliminación, la identificación de evidencia clínica o microbiológica de infección intrauterina o de alguna otra patología asociada, así como presencia de trabajo de parto, embarazos gemelares o complicados con alguna enfermedad metabólica. Las muestras fueron recolectadas y transportadas en condiciones de esterilidad hasta nuestro laboratorio en la Facultad de Medicina, UNAM.

## 1. Protocolo para la extracción de células coriodeciduales.

Para aislar a las células coriodeciduales, las membranas corioamnióticas se limpiaron y se eliminaron los coágulos de sangre con solución salina isotónica estéril. A continuación se realizó un raspado manual suave de la cara del corion con un raspador celular (Gibco, Invitrogen, USA) y se recolectó el material desprendido. Este paquete celular se pasó por un filtro estéril de 30 μm, que permitió obtener una suspensión celular libre de tejido. Las células separadas se pasaron después por un gradiente de densidad, utilizando el reactivo Lymphoprep (Axis-Shield, Oslo, Noruega) y se recuperaron todas las células localizadas en la interfase del gradiente correspondiente a la fracción enriquecida en linfocitos. Las células aisladas se lavaron con solución salina amortiguada con fosfatos (PBS) y se resuspendieron en medio de cultivo RPMI 1640 (Gibco, Invitrogen) complementado con 1% de hidrolizado de lactoalbúmina, 1mM de piruvato de sodio y mezcla de antibiótico/antimicótico (100 U penicilina + 100 μg de estreptomicina + 25.0 μg de anfotericina B/mL) y se dejaron incubar por 3 horas en botella de cultivo de 25cm$^2$ con el fin de eliminar a células con capacidad de adherencia. Al término del cultivo se recuperaron las células en suspensión, a los que denominamos leucocitos coriodeciduales, se contaron con hemocitómetro y se evaluó su viabilidad mediante el colorante azul tripano. Algunas células se procesaron para su inmunofenotipificación y otras se destinaron a su cultivo.

## 2. Protocolo para la extracción de leucocitos de la circulación placentaria.

Inmediatamente después de la cesárea, se colectó sangre de la circulación placentaria mediante drenado de los cotiledones de la placenta y se colocó en tubos heparinizados (Vacutainer-heparina sódica, Becton-Dickinson, Rutheford, NJ). Las muestras de sangre se sometieron a un gradiente de densidad con el reactivo Lymphoprep® (Axis-Shield, Oslo, Noruega), para obtener los leucocitos de acuerdo con el protocolo sugerido por los fabricantes. Una vez obtenidos los leucocitos, se contaron y se midió su viabilidad mediante el colorante vital azul tripano. Algunas células se procesaron para su inmunofenotipificación y otras se destinaron a su cultivo.

## 3. Cultivo de células coriodeciduales y leucocitos de la circulación placentaria.

Las células coriodeciduales aisladas y los leucocitos de sangre de placenta se mantuvieron en cultivo, para lo cual se sembraron $1x10^6$ células por pozo en placas de cultivo de 12 pozos en medio RPMI 1640 (Gibco, Invitrogen) complementado con 1% de hidrolizado de lactoalbúmina, 1mM de piruvato de sodio y mezcla de antibiótico/antimicótico (100 U penicilina + 100 μg de estreptomicina + 25.0 μg de anfotericina B/mL). Las células se mantuvieron en estufa de cultivo a 37ºC, en una atmósfera de 5% de $CO_2$ y 95% de aire, con humedad relativa a saturación. Se mantuvieron en estas condiciones hasta 72 horas y se recolectó el medio condicionado para su posterior análisis.

## 4. Caracterización de los leucocitos coriodeciduales y de la circulación placentaria

### 4.1 Inmunofenotipificación

Para describir a las poblaciones de células coriodeciduales y leucocitos placentarios aislados se utilizó la técnica de citometría de flujo. Se utilizaron anticuerpos monoclonales conjugados con fluorocromos para identificar a las diferentes subpoblaciones, incluidos en el IMK Kit (BD Bioscences, CA, USA); Cat. No.340504): leucocitos totales/$CD45^+$(clona 2D1-HLe-1), células NK/$CD16^+$(clona B73.1), $CD56^+$ (clona NCAM 16.2), linfocitos B/$CD19^+$ (clona SJ25C1), monocitos/macrófagos/$CD14^+$ (clona HCD14) y linfocitos/$CD3^+$ (clona SK7), $CD8^+$ (clona SK1) y $CD4^+$ (clona SK3).

Se identificó a las subpoblaciones de leucocitos dentro de la ventana de leucocitos totals/$CD45^+$ utilizando el equipo de la serie BD FACSCalibur (Becton, Dickinson and Company) equipado con Cell Quest Pro como programa de adquisición y análisis de datos de la Unidad de Citofluorometria del Instituto de Investigaciones Biomédicas, UNAM.

## 5. Evaluación funcional de los leucocitos coriodeciduales y de circulación placentaria

Se evaluó la capacidad funcional de los leucocitos coriodeciduales y placentarios mediante la secreción de citocinas, quimiocinas, de MMP-9 y MMP-3 asociadas en la degradación del tejido conectivo de las membranas corioamnióticas.

**5.1 Determinación de citocinas y quimiocinas**

Se colectó el sobrenadante de las células coriodeciduales y de los leucocitos de sangre placentaria en cultivo y se almacenaron a -80ºC hasta su utilización. La determinación de la concentración de citocinas y quimiocinas se utilizó el sistema de matriz de suspensión de perlas magnéticas MagPix (Luminex xMAP, Austin, TX, USA siguiendo el protocolo sugerido por el fabricante mediante el kit premezclado (Milliplex MAG, Millipore, Billerica, MA, USA) de 12 citocinas y 4 quimiocinas.

Sin embargo, en este trabajo sólo se presentan las citocinas que fueron significativamente diferentes entre compartimentos y que representan a los ambientes pro y antiinflamatorios; IL-6, IL-4; IL-1Ra; TNF-α, MIP-1α y MCP-1. El límite de detección se estableció en las concentraciones estándar bajas de <3.2 pg/ml para todas las citocinas.

**5.2 Zimografía en gelatina para determinar actividad de la MMP-9 y MMP-3**

La actividad gelatinolítica de los medios condicionados de las células aisladas de ambos grupos en estudio se determinó mediante geles/sustrato. Alícuotas de 0.5 µg de proteína de cada medio se sometió a electroforesis en geles de poliacrilamida al 8% con dodecil sulfato de sodio (SDS), co-polimerizados con gelatina tipo A de piel porcina o caseína (Sigma, St. Louis, MO, USA) al 5% en condiciones no desnaturalizantes utilizando un sistema con formato de minigel (Bio-Rad, Richmond, CA, USA). Posteriormente, se lavaron los geles con una solución de Tritón X-100 al 2.5% por 30 minutos. Se mantuvieron en incubación por 12 horas a 37ºC en una solución de Tris 50mM, NaCl 0.15 M y 0.1M de $CaCl_2$. Al término de la incubación los geles fueron teñidos con azul de Coomassie R-250. Las zonas de actividad enzimática aparecieron como bandas de lisis claras contra un fondo azul de sustrato no degradado. Se incluyó el sobrenadante de la línea celular de promielocitos U937 como marcador de actividad para MMP-9 y MMP-2. Se utilizó MMP-3 recombinante humana como control positivo en los geles sustrato de caseína.

**5.3 Cuantificación de la enzima total y activa de MMP-9**

La cuantificación específica de la forma total y activa de la MMP-9 en sobrenadantes de células coriodeciduales y leucocitos de sangre placentaria se realizó utilizando el sistema de ensayo de actividad Biotrak (General Electric Healthcare, Buckinghamshire, UK) siguiendo el protocolo sugerido por el fabricante. Con el fin de medir el contenido total de MMP-9, enzima unida se activó con acetato de p-aminofenilmercúrico. La concentración de la enzima total y activa MMP-9 en las muestras se reportó como ng de MMP-9 por µg de proteína.

## 6. Análisis estadístico

Se obtuvo estadística descriptiva (promedio, desviación y error estándar, mediana y rango) para cada variable. Se utilizaron las pruebas de Kologorov-Smirnoff y Shapiro-Wil para examinar la distribución y normalidad de los datos. Se utilizó la prueba de T-de Student para comparar las subpoblaciones de leucocitos coriodeciduales y los de placenta. Para comparar la secreción de citocinas y quimiocinas por los leucocitos coriodeciduales y de placenta se utilizó el análisis de mediciones repetidas. Se consideraron diferencias significativas con un valor de $p \leq 0.05$.

Todos los análisis estadísticos se realizaron utilizando el software SPSS v.20 (IBM Corporation, Armonk, NY, USA).

# RESULTADOS

## Aislamiento y caracterización de las células coriodeciduales

En el primer paso del aislamiento de leucocitos coriodeciduales se obtuvo una mezcla heterogénea de poblaciones celulares que se representa en la *figura 3,* compuesta al menos por células grandes con morfología de células deciduales y trofoblastos (*figura 3A)* acompañadas por células pequeñas con características de leucocitos *(figura 3B)*.

El uso de una metodología que comprende dos pasos; el primero mediante un gradiente de densidad seguido por la capacidad adherente de las células de linaje no leucocitario dio un rendimiento de 133,000 ± 3,500 leucocitos coriodeciduales por $cm^2$ de membrana fetal (n=18). Este método permitió la obtención de células de origen leucocitario con una pureza mayor del 80% y viabilidad mayor al 90%.

El fenotipo y la proporción de las subpoblaciones aisladas del espacio coriodecidual y de la circulación placentaria se muestran en la *tabla 1*. No se observaron diferencias significativas entre el compartimento coriodecidual y la circulación placentaria. Sin embargo, los linfocitos T ($CD3^+$) son los mas abundantes en ambos compartimentos seguidos de las células asesinas naturales. La *figura 4* muestra un ejemplo representativo del tipo de análisis que se realizó para la caracterización fenotípica de los leucocitos coriodeciduales y de placenta.

## Perfil inflamatorio secretado por los leucocitos coriodeciduales y placentarios

Leucocitos coriodeciduales mostraron un patrón de secreción de citocinas y quimiocinas distinto en comparación con los leucocitos placentarios. Los leucocitos coriodeciduales secretan un perfil proinflamatorio representado principalmente por IL-6 y TNF-α ($p<0.001$). En contraste, los leucocitos de sangre de placenta secretan un perfil anti-inflamatorio que se caracteriza por IL-4 e IL-1ra. La secreción de quimiocinas tales como MCP-1 es predominante por los leucocitos coriodeciduales ($p<0.001$) como se representa en la *figura 5*.

## Secreción y activación de proMMP-9 por las células coriodeciduales.

Los leucocitos placentarios y coriodeciduales secretan MMP-9 (92 kDa) en cultivo a partir de las 24 horas. La secreción total de MMP-9 por los leucocitos coriodeciduales aumentó significativamente de 24 a 72 h de cultivo ($p<0.01$; $n=15$). Por lo contrario, los leucocitos aislados de la circulación placentaria mostraron menor secreción de MMP-9 durante todo el tiempo de cultivo. Después de 72 h de cultivo, el total de MMP-9 secretada por los leucocitos coriodeciduales fue estadísticamente mayor que secretada por los leucocitos de sangre de placenta. La forma activa de MMP-9 (82 kDa) se observó a partir de 24 h y aumentó gradualmente hasta 72 h sólo en los medios obtenidos de leucocitos coriodeciduales. La forma activa de MMP-9 fue indetectable en el medio condicionado proveniente de leucocitos placentarios en el ensayo gelatinolítico *(figura 6A)*. La determinación cuantitativa de las formas totales y activas de MMP-9 también reveló un aumento gradual significativo en la forma activa de MMP-9 en los medios condicionados provenientes de leucocitos coriodeciduales; fenómeno no observado en los medios condicionados de leucocitos de sangre de placenta *(figura 6B)*.

Con el uso de la zimografía en caseína podemos observar que los leucocitos coriodeciduales también están secretando a la MMP-3 (54 kDa). La presencia de formas activas de MMP-9 coincide con el aumento en la secreción de MMP-3; lo que sugiere que existe una relación y que al menos una proporción de la activación de la MMP-9 podría estar explicada por la presencia de MMP-3.

# DISCUSIÓN

Al inicio y en el desarrollo del trabajo de parto al final de la gestación, involucra algunas de las etapas de la respuesta inflamatoria. La migración y arribo de diferentes subpoblaciones de leucocitos y la secreción de mediadores condicionan un microambiente específico en la coriodecidua (Thompson et al., 1999). Estudios previos han demostrado que la dilatación cervical correlaciona con el arribo de macrófagos y neutrófilos que secretan enzimas responsables de la degradación de la matriz extracelular en este tejido durante el trabajo de parto (Marvin et al., 2002). El arribo de leucocitos al miometrio, cérvix y a las membranas fetales coincide con la secreción de citocinas pro-inflamatorias como la IL-1, IL-6, IL-8, TNFα en estos tejidos durante el trabajo de parto (Young et al., 2010). Aunque aun no se conoce en su totalidad la red de señalización celular, ha sido aceptado que los leucocitos infiltrados en los compartimentos fetales y maternos son fuente de citocinas y de otros mediadores relacionados al trabajo de parto humano (Thompson et al., 1999; Keski-Nisula et al., 2000; Young et al., 2002; Gomez-Lopez et al., 2010).

En este trabajo se exploraron las propiedades funcionales de los leucocitos coriodeciduales aislados de las membranas fetales con el fin de conocer su participación en la secreción de moléculas asociadas a la degradación de la matriz extracelular de estos tejidos hacia final de la gestación. En el desarrollo de este proyecto se utilizaron membranas fetales que provinieron de mujeres que llegaron al término del embarazo y que fueron sometidas a cesárea, sin haber tenido algún signo de trabajo de parto. Seleccionamos este tipo de tejidos fetales porque representan las condiciones que prevalecen al término de la gestación. La evidencia científica sugiere que en este momento de la gestación muchos de los procesos asociados al inicio del trabajo de parto están presentes pero aun no son inducidos, lo que nos permite eludir la progresión del trabajo de parto.

Se aislaron leucocitos de membranas fetales provenientes de embarazos a término que están constituidos principalmente por una mezcla de linfocitos T, células NK y monocitos en una proporción similar a la de la circulación placentaria. Los leucocitos de sangre placentaria fueron nuestro modelo de comparación, ya que estas células provienen de espacios contiguos y son el mismo tipo celular.

Aunque no se encontraron diferencias en la proporción de las subpoblaciones en ambos compartimentos, observamos una mayor proporción de monocitos/macrófagos en la coriodecidua apuntando a la llegada de subconjuntos de leucocitos específicos a medida que se acerca el trabajo de parto. Esta conclusión está de acuerdo con las observaciones previas y nuevas que describen la infiltración de monocitos/macrófagos en los tejidos reproductivos cercano el trabajo de parto (Gomez-Lopez., 2010).

Recientemente hemos presentado pruebas que corroboran que la composición celular en la zona coriodecidual de las membranas fetales se modifica con el arribo de subpoblaciones de leucocitos (Gómez-López et al., 2011) donde la proporción de las células T ($CD3^+$), linfocitos T de memoria $CD4^+$ y $CD8^+$ aumentan al término de la gestación; siendo mayor su proporción en tejidos con trabajo de parto. La expresión y secreción de quimiocinas específicas para el reclutamiento de linfocitos T tales como CCL5 e IL-10 coincide con el término de la gestación. Hemos señalado la presencia de un grupo específico de linfocitos T ($CD4^+$ CD45RO) que representan a células T de memoria lo que sugiere que fueron generadas durante etapas tempranas del embarazo para establecer una tolerancia inmunológica necesaria para evitar el rechazo por parte de la madre hacia el feto o bien en embarazos anteriores. Sin embargo, hemos demostrado su participación durante las etapas tardías del embarazo y el trabajo de parto mediante la secreción de citocinas pro-inflamatorias y moléculas efectoras relacionadas a la degradación del tejido conectivo de las membranas fetales (Gómez-López et al., 2013).

La atracción específica y "homing" (reclutamiento) de los leucocitos al término de la gestación hacia la coriodecidua ha sido propuesto como el primer paso para el acondicionamiento de un microambiente proinflamatorio que resulta en la producción de mediadores que inducen el trabajo de parto (Estrada et al., 2005). La activación de la cascada inflamatoria puede desempeñar un papel importante en el trabajo de parto normal y patológico, así como en el parto prematuro y la corioamnioitis. A pesar de que se han realizado estudios para evaluar las concentraciones de citocinas en diferentes compartimentos tales como el cordón umbilical, líquido amniótico y sangre periférica materna recolectada en diferentes etapas del embarazo y el parto; el papel inmunobiológico de las citocinas inflamatorias al final de la gestación y en el parto en condiciones estériles siguen sin ser elucidados en su totalidad.

En este trabajo se demostró que en condiciones de cultivo, los leucocitos coriodeciduales secretan un perfil de moléculas efectoras caracterizado por mayor secreción de citocinas proinflamatorias (TNF-a e IL-6) y menor secreción de citocinas antinflamatorias (IL-10 y IL-1ra). El perfil de secreción de moléculas por los leucocitos coriodeciduales esta representado por citocinas proinflamatorias lo que coincide con otros autores acerca de que el progreso del trabajo de parto está asociado con eventos proinflamatorios ((Young et al., 2002; Thompson et al., 1999). En contraste, los leucocitos de la circulación placentaria mostraron un perfil básicamente representado por citocinas anti-inflamatorias.

Por otro lado, los leucocitos coriodeciduales contienen al menos una subpoblación con la capacidad de activación y secreción de MMP-9 en comparación con las células equivalentes de compartimentos cercanos. Los leucocitos coriodeciduales incrementaron exponencialmente su capacidad para secretar MMP-9, tres veces más que otras líneas celulares de referencia tales como U937 (Watanabe et al., 1995) o cantidades equivalentes de líneas de cáncer metastásico (Hoskins et al., 2011). A pesar de que no hemos identificado la subpoblación responsable de la secreción de MMP-9, la producción de esta enzima por poblaciones de leucocitos situados en las proximidades de las membranas fetales hace de estas células candidatas para promover la degradación de estos tejidos al término de la gestación.

El cambio observado en la capacidad para la secreción de MMP-9 por los leucocitos extraídos en cultivo sugiere la existencia de un mecanismo para el control negativo de la expresión de MMP-9 en el interior de las membranas corioamnióticas en este momento de la gestación. El aumento de la forma activa de MMP-9 durante el cultivo de leucocitos coriodeciduales coincide con la presencia de MMP-3, también secretada por estos leucocitos. Varios estudios *in vitro* han documentado que la MMP-3 es el eje principal en la activación fisiológica de la MMP-9 (Vandooren et al., 2013). Sin embargo, la secreción de MMP-3 por los leucocitos coriodeciduales sólo es capaz de activar una fracción de la MMP-9 secretada, manteniéndose la gran mayoría de la enzima de manera inactiva. La latencia de la MMP-9 puede permitir la acumulación de enzimas líticas para el momento de la ruptura de las membranas. En un trabajo previo se demostró la existencia de un complejo macromolecular compuesto por varias MMPs, asociadas a las membranas corioamnióticas teniendo la capacidad de actuar como una verdadera reserva de estas enzimas proteolíticas hasta el momento de la ruptura (Meraz-Cruz et al., 1999).

Se postula que la total activación de la MMP-9 y otras MMPs se alcanzará a lo largo del proceso de trabajo de parto activo provocando la pérdida de la resistencia mecánica y la ruptura de las membranas fetales pudiendo ocurrir bajo condiciones fisiológicas o en la ruptura de las membranas fetales pretérmino.

Con base a los resultados se demostró que los leucocitos coriodeciduales aislados de membranas fetales a término son funcionalmente diferentes de leucocitos de la circulación placentaria y debido a su capacidad de secreción de MMP-9 y de moléculas pro-inflamatorias, pueden estar asociadas en el desencadenamiento del proceso de la ruptura de las membranas fetal al término de la gestación humana.

Estos resultados suman significado funcional de observaciones pasadas y actuales donde describe el arribo de leucocitos a los tejidos reproductivos cercano el trabajo de parto. Nuestro grupo recientemente proporcionó evidencia que apoya que la composición celular coriodecidua se modifica selectivamente a término la gestación con la llegada de los subgrupos de linfocitos específicos, principalmente el arribo de linfocitos T y granulocitos, algunos de ellos expresan MMP-9, IL-1β y TNF-α. Nuestros hallazgos *ex vivo* evaluando leucocitos coriodeciduales en cultivo son complementarios a los reportados *in vivo*. Utilizando técnicas histológicas se muestra la asociación de las células inmunológicas de las diferentes zonas de las membranas fetales (zonas en relación al sitio de ruptura) con mediadores pro-inflamatorios tales como IL-1β y TNF-α; así como moléculas efectoras como a la MMP-9 relacionada a la degradación de las membranas fetales.

La quimiotaxis y el reclutamiento de leucocitos en la zona coriodecidual de las membranas fetales hacia el final de la gestación condicionan un microambiente pro-inflamatorio asociado al trabajo de parto. Las quimiocinas tales como CCL3, CCL2, IL-8 y CCL5 se incrementan en el líquido amniótico y este aumento correlaciona con dilatación cervical y el número de leucocitos en los tejidos reproductivos con trabajo de parto a término (Romero et al., 1994; Esplin *et al.*, 2003; Athayde *et al.*, 1999). CCL3, IL-6 y CCL2 son secretadas por los leucocitos coriodecidaules, y estas señales pueden atraer y activar a otros linfocitos y monocitos.

De acuerdo con nuestra hipótesis, una vez iniciado el arribo de leucocitos al espacio coriodecidual modulado por quimiocinas, la activación de la cascada inflamatoria por un modulador aún no identificado se traducirá en la liberación local y masiva de mediadores, incluyendo IL-1ß, TNF-α e IL-6 donde los leucocitos coriodeciduales pueden ser una fuente importante de estas señales. Estas citocinas se han propuesto como una señal primaria, que ejercerán un efecto sobre las células locales lo que resulte en la producción de moléculas efectoras que amplifiquen el evento inflamatorio asociado al trabajo de parto. A pesar de que no podemos integrar totalmente el diálogo molecular resultante entre los leucocitos y las células locales en la coriodecidua, los datos experimentales nos permite sugerir que las citocinas secretadas por los leucocitos coriodeciduales como es el caso de TNF-α podrían estimular la expresión y la secreción de metaloproteasas y ser el mecanismo responsable en nuestras condiciones experimentales.

En conclusión, nuestros resultados demuestran que los leucocitos coriodeciduales aislados de las membranas fetales a término de la gestación son funcionalmente diferentes de los leucocitos residentes en otros compartimentos, y pueden colaborar para modular el microambiente ligado a la inducción y progresión del trabajo de parto al término de la gestación humana.

# CONCLUSIONES

- Los leucocitos provenientes del espacio coriodecidual secretan un patrón de citocinas diferente a los leucocitos provenientes de la circulación placentaria; a pesar de compartir el mismo tipo celular y pertenecer a compartimentos contiguos.

- Los leucocitos coriodecidaules secretan en mayor concentración citocinas pro-inflamatorias y quimiocinas; y en menos concentración citocinas anti-inflamatorias. Esta respuesta inflamatoria ha sido asociada al inicio y progresión del trabajo de parto humano.

- Los leucocitos coriodeciduales presentan capacidades funcionales asociadas al término de la gestación y trabajo de parto. Secretan y activan MMP-9 significativamente mayor que los leucocitos de sangre placentaria.

# PERSPECTIVAS

- Caracterización celular y funcional del microambiente coriodecidual de las membranas fetales con trabajo de parto y membranas fetales pretérmino.

- Caracterización celular de los leucocitos coriodecidaules con marcadores asociadados a la activación y función de linfocitos T.

- Caracterización de la comunicación intercelular en el ambiente coriodecidual al término de la gestación.

# FIGURAS

## FIGURA 3.

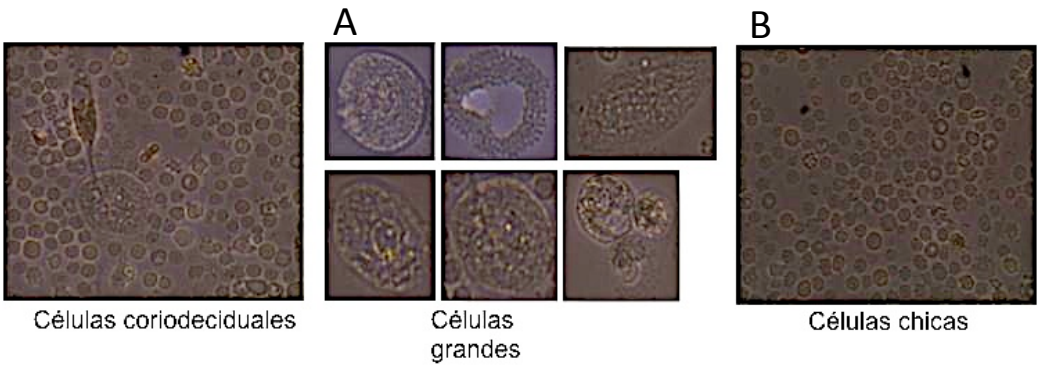


**Figura 3.** Micrografías de células coriodeciduales. En el panel (A) se muestran células grandes, Panel (B) se observan células pequeñas.

## FIGURA 4.

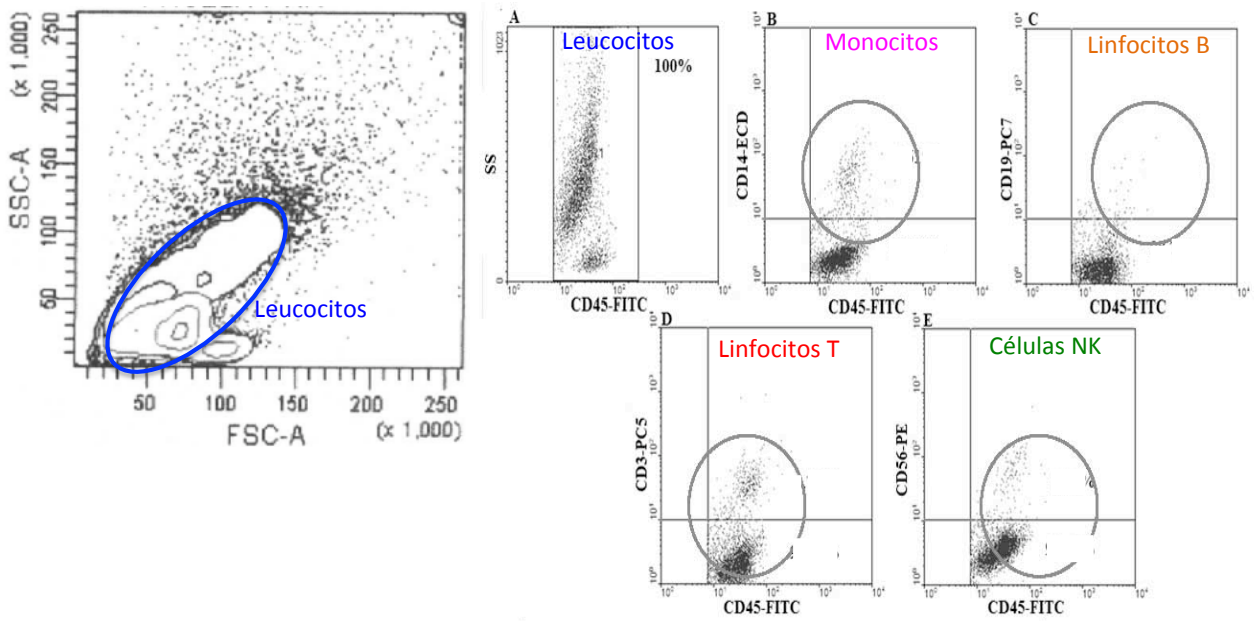


**Figura 4.** Imágenes de la inmunofenotipificación de las células obtenidas de la coriodecidua, utilizando marcadores de leucocitos. En el panel A se muestra la distribución de todas las poblaciones de leucocitos (CD45+). En el panel B se muestra la proporción de monocitos (CD45+.CD14+). En el panel C la subpoblación de linfocitos B (CD45+,CD19+). En el panel D la proporción de linfocitos T (CD45+,CD3+) y en el panel E, la proporción de células NK (CD45+,CD56+). La mayor parte de las células CD45+, correspondieron a granulocitos (45-52%) y el resto correspondieron a células grandes mezcladas negativas a CD45.

FIGURA 5

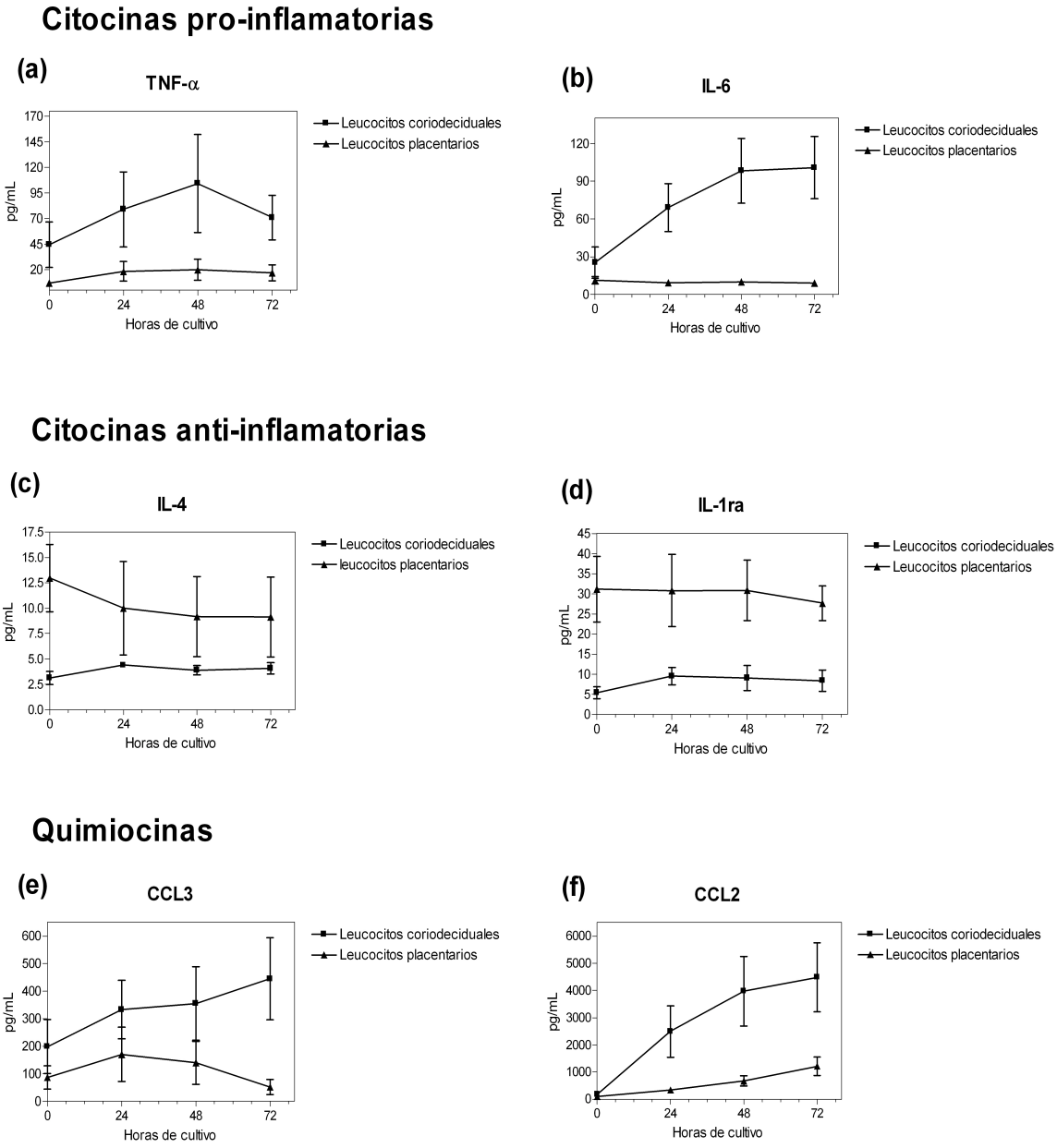


**Figura 5.** Caracterización del perfil de inflamación secretado por leucocitos coriodeciduales y placentarios.

Leucocitos coriodeciduales y placentarios fueron aislados y cultivados hasta 72 horas. La cuantificación de citocinas y quimiocinas se representan en paneles individuales: **(a)** Factor de necrosis tumoral-α (TNF-α), p=0.009; **(b)** Interleucina-6 (IL-6), p=0.000; **(c)** Interleucina-4 (IL-4), p=0.007; **(d)** receptor antagonista de IL-1 (IL-1ra), p=0.000 **(e)** Proteína inflamatoria-1α (MIP-1α/CCL3) ), p=0.005; **(f)** Proteína quimiotáctica de monocitos 1 (MCP-1/CCL2), p=0.006. Los puntos en la gráfica representan el promedio DE de 5 experimentos independientes en duplicado por grupo. Análisis multivariado muestran diferencias entre grupos; $p<0.05$.

FIGURA 6

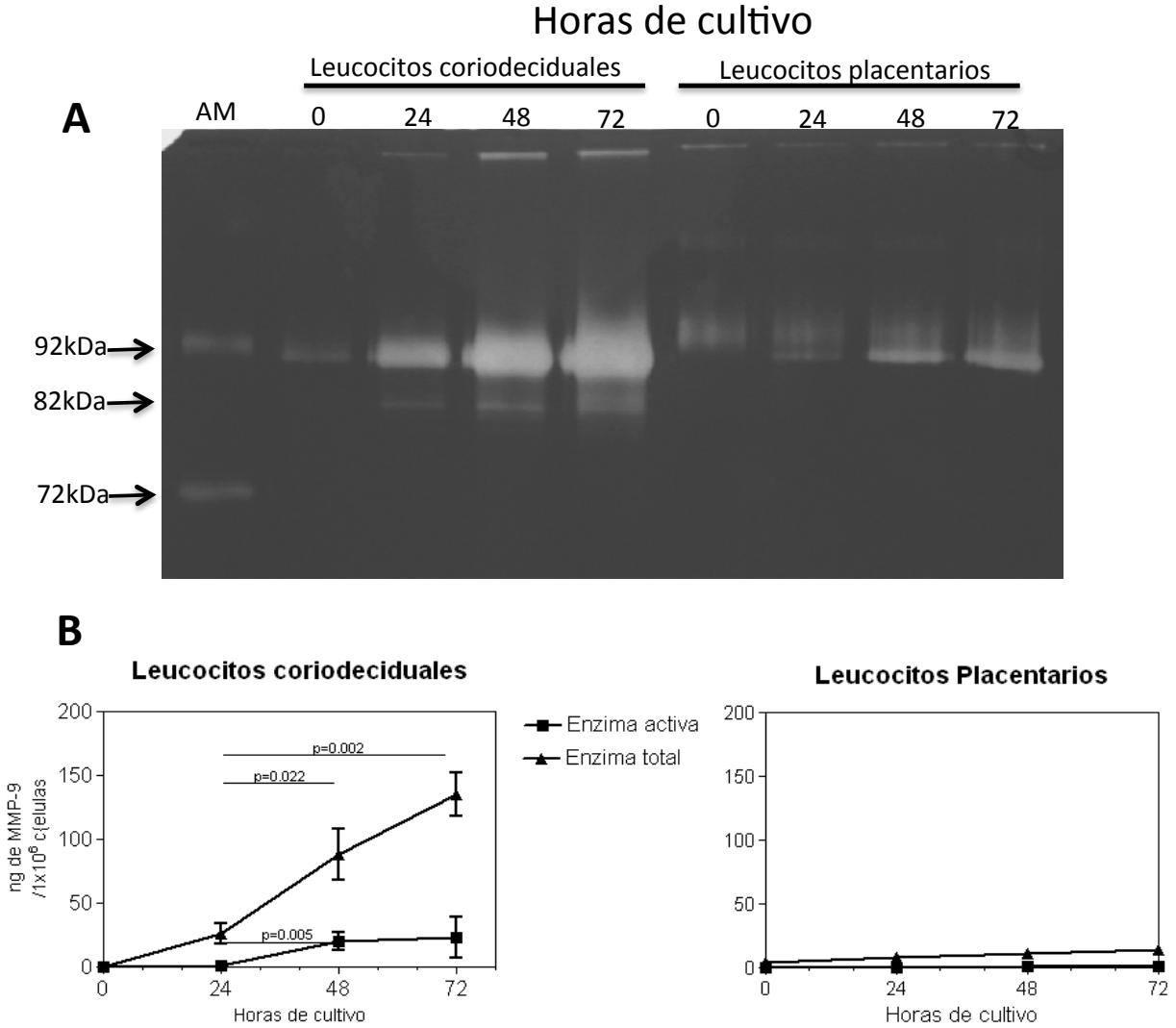


**Figura 6.** Actividad gelatinolítica y cuantificación de las formas activas y totales de MMP-9.

**6A**. El medio condicionado de leucocitos coriodeciduales y de placenta cultivados por 24, 48 y 72 horas fue analizado por zimografía en gelatina. El corrimiento electroforético (bandas claras) muestra la forma inactiva de MMP-9 (92kDa) y la forma active (82kDa) respectivamente. (n=7). El medio condicionado de la línea celular U937 se utilizó como marcador de actividad. **6B**. La formas activas y totales de MMP-9 en estos mismos medios condicionados fueron analizados por el sistema ELISA. Cada punto en la gráfica representa el promedio ± SD (n=3).

FIGURA 7

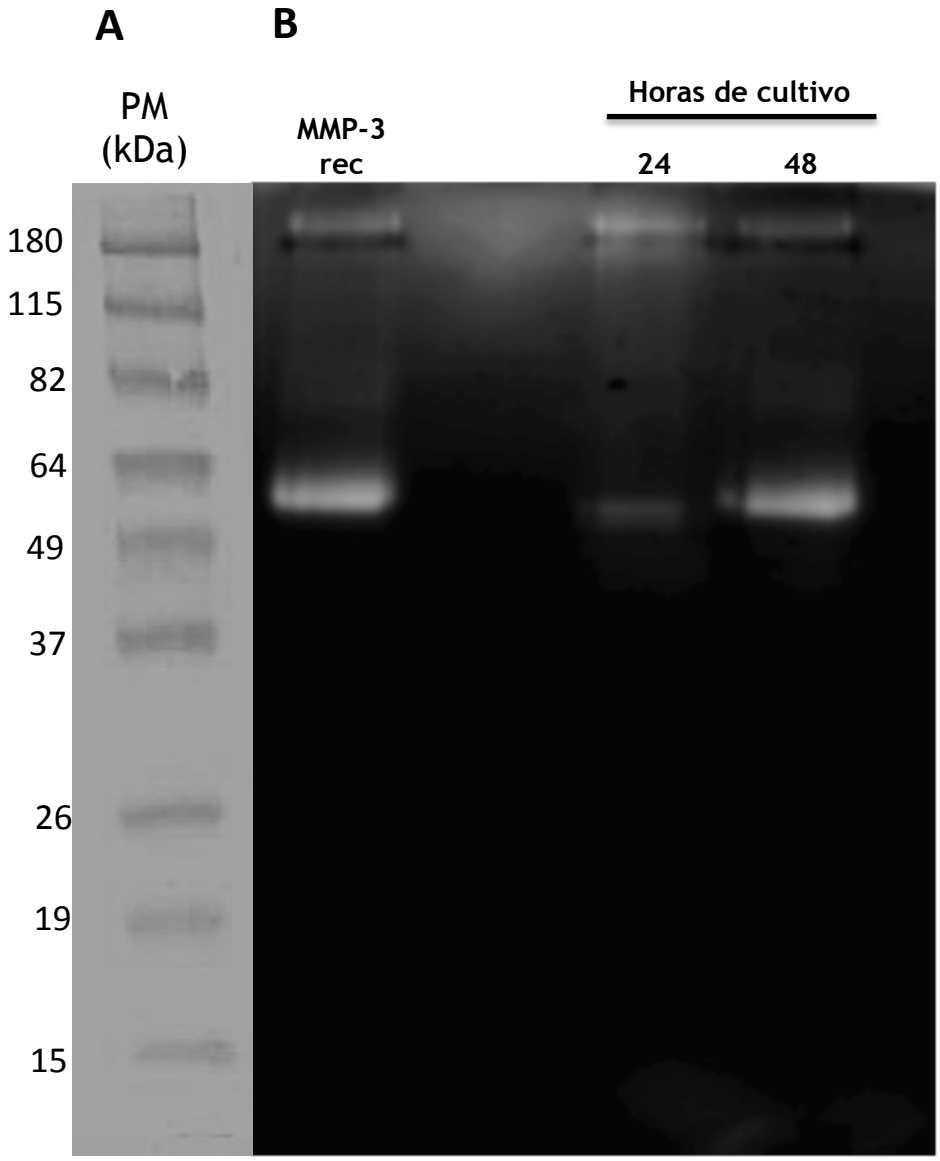


**Figura 7.** Identificación de MMP-3 secretada por los leucocitos coriodeciduales.
Leucocitos coriodeciduales fueron aislados y cultivados hasta 48 horas. Se identifico la presencia de MMP-3 mediante geles/sustrato con caseína. **(A)** PM= peso molecular en gel de acrilamida. **(B)** En el primer carril se observa la actividad por MMP-3 recombinante humana 20ng/mL. En el tercer y cuarto carril se observa la presencia de MMP-3 secretada por leucocitos coriodeciduales.

# TABLAS

## TABLA 1

**Porcentaje de las supoblaciones de leucocitos coriodeciduales y de la circulación placentaria.**

| Subpoblación | Coriodecidua (n=7) | Circulación Placentaria (n=7) |
|---|---|---|
| Linfocitos T (CD3+) | 35 (30.7-42.3) | 35.5 (32.3-38.5) |
| Linfocitos T (CD4+) | 27.8 (27.3-28.3) | 26.9 (14.6-24.7) |
| Linfocitos T (CD8+ ) | 11.1 (4.2-18.0) | 14.7 (11.7-17.8) |
| Células NK(CD56+) | 13.9 (12.7-15.1) | 10.5(10.33-10.7) |
| Monocitos/macrófagos (CD14+) | 8.5 (8.0-9-0) | 4.9 (4.9-5.0) |
| Linfocitos B (CD19+) | 1.8 (0.9-2.8) | 1.9(1.47-2.4) |

Tabla 1. Comparación del porcentaje de leucocitos coriodeciduales y dela circulación placentaria mediante citometria de flujo. Se representa media (mínimo-máximo).

El fenotipo de leucocitos se analizó dentro de una región positiva para CD45.

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# APÉNDICE

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- **Castillo-Castrejon M,** Meraz-Cruz N, Gomez-Lopez N, Flores-pliego A, Beltran-Montoya J, Viveros-Alcaraz M, Vadillo-Ortega F. Choriodecidual cells from term human pregnancies show distintive functional properties related to the induction of labor. Am J Reprod Immunol. (2014)71:86-93. DOI: 10.1111/aji.12179.

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## RESÚMENES EN CONGRESOS

- Marzo 2013. Presentación en poster en Congreso. 60th Annual Scientific Meeting organizado por la Society for Gynecologic Investigation. Orlando, FL. U.S.A. Trabajo: "A Differential Pattern of Functions Is Exhibited by Leukocytes Isolated from the Choriodecidua at Term of Human Gestation. Autores: **Marisol Castillo-Castrejón,** Noemí Meraz-Cruz, Jorge Beltrán-Montoya, Martín Viveros Alcaraz, Felipe Vadillo-Ortega.

- Septiembre 2012. Presentación oral en Congreso. V Congreso Internacional de Aplicaciones en Citometría de Flujo. Facultad de Medicina. UNAM. Mexico D.F. Capitulo de Citometría de flujo de la Sociedad Mexicana de Inmunología. Trabajo Los leucocitos coriodeciduales condicionan un microambiente intrauterino de activación del trabajo de parto humano. Autores: **Marisol Castillo-Castrejón**, Noemí Meraz-Cruz, Arturo Flores-Pliego, Jorge Beltrán-Montoya, Felipe Vadillo-Ortega.

- Octubre 2010. Presentación en poster en International Federation of Placenta Associations Meeting 2010. Santiago, Chile. Trabajo de investigación básica. Trabajo: Traffiking of MMP-9 through amniochorion is associated to term human gestation. Autores: **Marisol Castillo-Castrejón**, Noemí Meraz-Cruz, Felipe Vadillo-Ortega.

ORIGINAL ARTICLE

# Choriodecidual Cells From Term Human Pregnancies Show Distinctive Functional Properties Related to the Induction of Labor

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**Keywords**
Amniochorion, choriodecidua, fetal membranes, inflammation, labor

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## Problem
Human parturition is associated with an intrauterine pro-inflammatory environment in the choriodecidua. Evidence that some mediators of this signaling cascade also elicit responses leading to labor prompted us to characterize the cellular sources of these mediators in the human choriodecidua.

## Method of study
Leukocyte-enriched preparations from human choriodecidua (ChL) and intervillous placental blood leukocytes (PL) were maintained in culture. Secretions of inflammatory cytokines, chemokines, and MMP-9 were documented. Leukocyte phenotype of ChL and PL was determined by flow cytometry using specific fluorochrome-conjugated antibodies.

## Results and Conclusions
ChL showed a distinct pro-inflammatory secretion pattern of cytokines and chemokines when compared with PL, including higher amounts of TNF-α and IL-6, and decreased secretions of IL-4 and IL-1ra. ChL also secreted more MIP-1α and MCP-1 and MMP-9 than PL. No significant differences were found in leukocytes subsets between compartments. Based on our findings, we propose that ChL isolated from fetal membranes at term are functionally different from PL and may collaborate to modulate the microenvironment linked to induction and progression of human labor.

## Introduction

The pathway of parturition is a complex process involving anatomical, biochemical, endocrinological, and immunological factors.[1] Human labor appears as a sequence of events initiated by myometrial contractions, then the cervix ripens, the fetal membranes rupture, and the fetus and placenta are expelled.[2] The mechanisms underlying the onset and progression of normal spontaneous labor remain unclear.

Increasing evidence shows that some components of the inflammatory pathway are involved in normal term labor.[3–5] The choriodecidual microenvironment during late gestation and during labor experiences functional modifications that include the active secretion of cytokines and chemokines, which results in the recruitment and activation of certain

leukocytes subpopulations.[6–11] Identified components of this network include pro-inflammatory and anti-inflammatory cytokines and chemokines.[8–10,12–18]

These mediators may act as primary paracrine and autocrine signals, eliciting the local secretion of secondary mediators, such as prostaglandins that act as uterotonics,[19] and matrix metalloproteinases (MMPs), such as 92 kDa type IV collagenase (MMP-9), which in turn is able to degrade the main extracellular matrix components of fetal membranes and promote their rupture.[20–23]

New evidence and old evidence support that the phenotype of the leukocytes in the choriodecidual microenvironment changes during labor at term, and T lymphocytes increase significantly in this site.[10,14,18] The arrival of a specific subset of lymphocytes may be linked to the choriodecidual activation observed at the term of gestation. In this article, we analyzed the contribution of choriodecidual lymphocytes to the secretion of cytokines, chemokines, and MMP-9, comparing the secretions of equivalent lymphocytes isolated from intervillous placenta blood, a nearby compartment.

## Materials and methods

### Patients and Biological Samples

Placentae and amniochorion samples were obtained from women at term gestation (38–40 weeks) undergoing indicated cesarean section without active labor and without clinical or microbiological infection determined by culture. Patients were recruited at Hospital Materno Infantil Inguarán, Secretaría de Salud del D.F. in México City, México. Participating women gave their informed consent, and the project was accepted by the local IRB (Register No. 101-010-08-09). All procedures described below were carried out within the first hour of collection of samples and under sterile conditions.

### Isolation and Culture of Placental Leukocytes

Leukocytes were obtained from intervillous placental blood (named placenta leukocytes or PL; $n = 9$) as follows. After the placenta was delivered, intervillous blood was drained out by manually compressing the cotyledons and recovered in sterile tubes containing heparin as anticoagulant (Becton-Dickinson, Franklin Lakes, NJ, USA). PL were isolated by density gradient using Lymphoprep (Axis-Shield, Oslo, NOR). Placental blood leukocytes were then cultured in RPMI 1640 culture media supplemented with 0.2% lactalbumin hydrolysate, 1% sodium pyruvate, and 1% antibiotic–antimycotic (RPMI/HLA; Gibco BRL, Grand Island, NY, USA). Cell viability was confirmed to be over 95% by staining with trypan blue. Lastly, PL ($1 \times 10^6$) were placed in 12-well plates (Corning Costar, NY, USA) with 700 μL of RPMI/HLA and incubated for 24, 48, and 72 hr at 37°C with 95% air/5% $CO_2$.

### Isolation and Culture of Choriodecidual Cells

Fetal membranes ($n = 9$) were collected after delivery and immediately washed to eliminate blood clots with saline isotonic solution in sterile conditions. Choriodecidual cells were obtained by gently scraping the chorionic side with a cell scraper (Sarstedt, Nümbrecht, Germany). Choriodecidual cell suspension was washed with phosphate-buffered solution [(PBS); 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2)] (Life Technologies, Carlsbad, CA, USA) and filtered with a MACS pre-separation filter (30 μm) to eliminate tissue fragments (Miltenyi Biotec, Bergisch Gladbach, Germany).[18] Choriodecidual cells were separated in Lymphoprep as described above. Gradient interphase including leukocytes was transferred into 25 $cm^2$ plastic flasks (Corning Costar, NY, USA) and incubated for 3.0 hr at 37°C in 95% air/5% $CO_2$. Non-adherent choriodecidual cells, choriodecidual leukocyte-enriched preparation (ChL), hereinafter, ($1 \times 10^6$ cells) were placed in 12-well plates (Corning Costar, NY, USA) in RPMI/HLA and incubated for 24, 48, and 72 hr at 37 °C with 95% air/5% $CO_2$. Cell viability was confirmed to be over 95% by trypan blue staining.

### Measurement of Cytokines and Chemokines in Cell Supernatants

After cell culture, ChL and PL conditioned media were collected and stored at −80°C until use. Samples were analyzed on a MAGPIX magnetic bead suspension array system (Luminex xMAP, Austin, TX, USA) using the multiplex sandwich immunoassay as per the manufacturer's protocols. A premixed human cytokine/chemokine magnetic bead assay kit (Milliplex MAG, Millipore, Billerica, MA, USA) was used to determine the concentration of TNF-α, IL-6, Il-4, IL-1ra, MIP-1α, and MCP-1. Other cytokines/chemokines were excluded using previous assays.

All samples were performed in one-plate run modus. The detection limit was set at the lowest standard concentration for each cytokine (pg/mL): TNF-α (0.7), IL-6 (0.9), IL-4 (4.5), IL-1ra (8.3), MIP-1α (2.9), and MCP-1 (1.9).

### Flow Cytometry for Leukocyte Subsets

Leukocytes subsets were characterized in ChL and PL using the BD Multitest IMK kit following the manufacturer's protocol (BD Biosciences, CA, USA; Cat. No. 340504): total leukocytes/CD45$^+$(clone 2D1-HLe-1), NK cells/CD16$^+$ (clone B73.1), CD56$^+$ (clone NCAM 16.2), B cells/CD19$^+$ (clone SJ25C1), and monocytes/macrophages/CD14$^+$ (clone HCD14), and subsets of T cells/CD3$^+$ (clone SK7), CD8$^+$ (clone SK1), and CD4$^+$ (clone SK3). Leukocyte subsets were analyzed within the CD45$^+$ gate using a FACSCalibur flow cytometer, and data analysis was performed by BD Cell Quest software (BD Biosciences, CA, USA).

### Zymography for Gelatinolytic Activity of MMP-9

Gelatinase activity in culture media was determined by SDS–PAGE containing 1% gelatin under non-denaturing conditions as described previously.[21] Culture supernatants (0.5 μg of total protein) were loaded into each well. Enzymatic activity standards for MMP-2 and MMP-9 were included using conditioned media on the U-937 promyelocyte cell line.[24]

### Quantification of Total and Active Forms of MMP-9

Specific quantification of active and total MMP-9 in culture supernatants of choriodecidual and peripheral leukocytes was carried out using the Biotrak MMP-9 Activity Assay System (General Electric Healthcare, Buckinghamshire, UK) following the protocol suggested by the manufacturer. To measure the total MMP-9 content, bound enzyme was activated with p-aminophenylmercuric acetate. The concentration of total and active MMP-9 in the samples is reported as nanograms (ng) of MMP-9 per μg of protein. Protein was measured by Bradford's method.[25]

### Statistical Analysis

For each variable, descriptive statistics (mean, standard deviation, standard error, median, and range) were obtained, and the data distribution was tested for normality using the Kolmogorov–Smirnoff and Shapiro–Wilk tests. Student's *t*-test was performed to compare leukocytes subsets between ChL and PL. A $P$ value $\leq 0.05$ was considered to be statistically significant. Two-way analysis of variance using repeated measurement model was used to compare cytokines/chemokines concentrations in the culture media from ChL and PL. Differences with $P \leq 0.05$ were considered statistically significant. All statistical analyses were carried out using SPSS, version 20 software (IBM Corporation, Armonk, NY, USA).

## Results

The two-step method, using a density gradient followed by selection by plastic adherence, yielded in 1,33,000 ± 3,500 choriodecidual leukocytes per cm$^2$ of fetal membranes ($n$ = 18). According to the flow cytometry data, this method also allowed enriching and purifying (≥80%) choriodecidual leukocytes. Flow cytometry analysis revealed that T lymphocytes and natural killer cells were the major subsets in the ChL and PL preparations (Table I).

**Table I** Percentages of Leukocytes Subsets in Choriodecidua and Intervillous Placental Blood

| Subset | Choriodecidua ($n$ = 7) | Intervillous placental blood ($n$ = 7) |
|---|---|---|
| Total T lymphocytes (CD3$^+$) | 35 (12.7–15.1) | 35.5 (32.3–38.5) |
| CD4$^+$ T lymphocytes | 27.8 (27.3–28.3) | 26.9 (14.6–24.7) |
| CD8$^+$ T lymphocytes | 11.1 (4.2–18.0) | 14.7 (11.7–17.8) |
| NK cells (CD56$^+$) | 13.9 (12.7–15.1) | 10.5 (10.33–10.7) |
| Monocytes/macrophages (CD14$^+$) | 8.5 (8.0–9.0) | 4.9 (4.9–5.0) |
| B lymphocytes (CD19$^+$) | 1.8 (0.9–2.8) | 1.9 (1.47–2.4) |

Evaluation of leukocytes subsets found in choriodecidua and placental blood analyzed by flow cytometry. No significant differences were found between compartments. Median values (minimum–maximum) are reported. The phenotypes of leukocytes subsets were analyzed with in a CD45$^+$ region. Choriodecidual leukocytes refer to non-adherent cells.



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Choriodecidual leukocytes showed a distinct secretion pattern of cytokines and chemokines when compared with intervillous placental blood leukocytes (Fig. 1). Overall, choriodecidual leukocytes secreted a pattern of pro-inflammatory cytokines, which included higher amounts of TNF-α and IL-6 along the period of incubation ($P < 0.001$), and decreased secretions of IL-4 and IL-1ra, compared with intervillous placental blood leukocytes. Choriodecidual leukocytes also secreted more MIP-1α and MCP-1 than placental blood leukocytes ($P < 0.001$) (Fig. 1).

Placental and choriodecidual leukocytes secreted pro-MMP-9 (92 kDa) in culture after 24 hr as revealed by zymography. The total MMP-9 secretion of the choriodecidual leukocytes significantly increased from 24 to 72 hr of culture ($n = 15$; $P < 0.01$). Discrete and constant secretion of proMMP-9 was observed by placental leukocytes during the entire culture period. The active form of MMP-9 (82 kDa) was present from 24 hr and increased after 48 and 72 hr only in the media of choriodecidual leukocytes. Barely visible amounts of active MMP-9 were identified in the media culture of leukocytes isolated from placental blood during the culture period (Fig. 2a).

Quantitative determination of the total and active forms of MMP-9 also revealed a gradual significant increase in the active form of MMP-9 in choriodecidual leukocytes from 24 to 72 hr of culture ($n = 8$; $P < 0.01$). After 72 hr of culture, total secreted MMP-9 by choriodecidual leukocytes was statistically greater than the amount secreted by intervillous placental blood leukocytes ($P = 0.003$). The active form of MMP-9 was barely detectable in the media culture of placental leukocytes (Fig. 2b).

## Discussion

Growing evidence suggests that some stages of the inflammatory response are present during initiation and/or progression of human parturition.[14,26–28] These changes include the conditioning of a specific microenvironment in the choriodecidua characterized by migration and homing of specific populations of leukocytes and secretion of mediators resembling an intrauterine pro-inflammatory milieu.[8–10,15,29]

In this article, we explored the functional properties of a choriodecidual leukocyte-enriched preparation isolated from fetal membranes, from pregnancies of at least 38 weeks of gestation in which the mothers underwent cesarean section without signs of spontaneous labor. We selected these tissues because they represent the prevalent conditions at the end of gestation, and evidence suggests that at this time of gestation, many of the processes associated with initiation of labor are present. To assess the specific functional properties of choriodecidual leukocytes, we compared these cells with the leukocytes isolated from intervillous maternal peripheral leukocytes of the same women.

Leukocytes isolated from term choriodecidua consisted mainly of a mix of T lymphocytes, NK cells, and monocytes in a proportion similar to that in intervillous maternal peripheral blood. However, these cells showed remarkably different functional properties compared with equivalent subsets isolated from placenta circulating blood. In culture, choriodecidual leukocytes secreted a combination of modulators characterized by increased amounts of pro-inflammatory cytokines, including TNF-α and IL-6, and decreased amounts of anti-inflammatory signals (IL-10 and IL-1rα). The balance of this network of signaling molecules is clearly inclined to pro-inflammation. In addition, choriodecidual leukocytes secreted chemokines and active MMP-9. Based on these findings, we propose that term choriodecidua contains a potential cellular source of pro-inflammatory mediators and the enzymatic machinery required for amniochorion extracellular matrix degradation associated with normal delivery at the end of gestation. Characterization of the specific subsets of cells participating in the secretion of these compounds is currently under way in our laboratory.

These findings add functional meaning to old and new observations describing the infiltration of leukocytes in reproductive tissues near the time of labor.[10,14,18,27,28,30] Our group recently provided evidence supporting that the choriodecidua cellular composition is actively and selectively modified at gestational term with the arrival of specific lymphocyte subsets, some of them expressing MMP-9, IL-1β, and TNF-α.[10,17]Our findings using *in vitro*-cultured choriodecidual leukocytes are also complementary to the previously reported *in vivo* presence of leukocytes in the choriodecidua expressing pro-inflammatory mediators, such as those described in this article, in human tissues experiencing labor.[10,18,31]

Specific chemo-attraction and homing of leukocytes to term gestation choriodecidua have been proposed as the first step for conditioning a pro-inflammatory

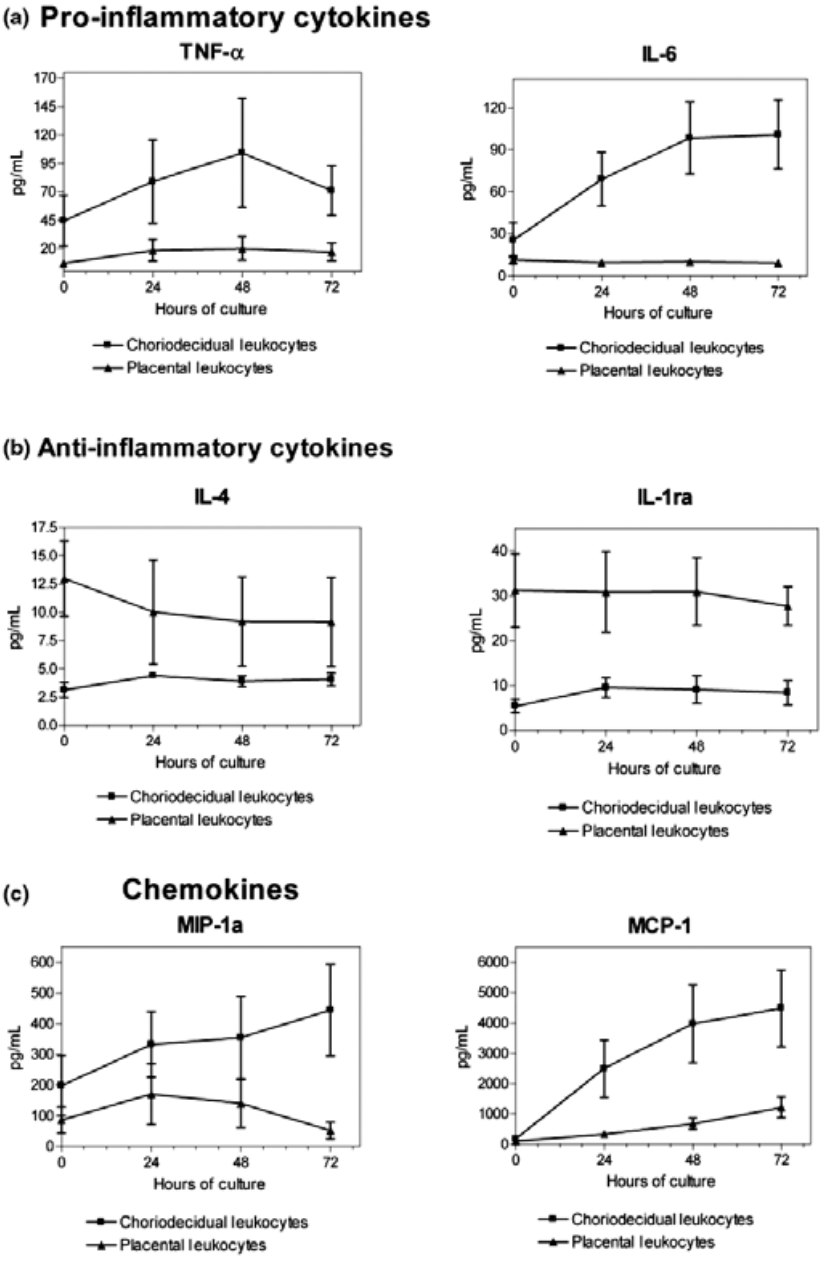


**Fig. 1** Characterization of the inflammatory profile of choriodecidual and intervillous placental blood leukocytes. Choriodecidual and intervillous placental blood leukocytes were obtained and cultured up to 72 hr. Quantification of cytokines and chemokines are represented in individual panels: (a) Tumor necrosis factor α (TNF α), $P = 0.009$; (b) Interleukin-6 (IL-6), $P = 0.000$; (c) Interleukin-4 (IL-4), $P = 0.007$; (d) IL-1 receptor antagonist (IL-1ra), $P = 0.000$ (e) inflammatory protein α (MIP-1α), $P = 0.005$; (f) monocyte chemotactic protein 1 (MCP-1), $P = 0.006$. Data are shown as means ± S.D. of determination of five independent experiments in duplicate per group. Multivariate analysis revea.led significant differences between groups; $P < 0.05$.

microenvironment resulting in the production of mediators for the induction of labor at term pregnancy.[13,32–34] Chemokines such as MIP-1α, MCP-1, IL-8, and RANTES are increased during labor in amniotic fluid, and this increase correlates with cervical dilation[33] and the number of leukocytes in reproductive tissues at term labor.[35–37] MIP-1α, IL-6, and MCP-1 are secreted by choriodecidual leukocytes,[8,31] and these signals may attract and activate additional lymphocytes and monocytes, among other leukocytes.[34]

According to the current hypothesis, once homing of leukocytes to the choriodecidua is under way, activation of the inflammatory cascade by a non-identified modulator will result in the massive local liberation of mediators, including IL-1β, TNF-α, and


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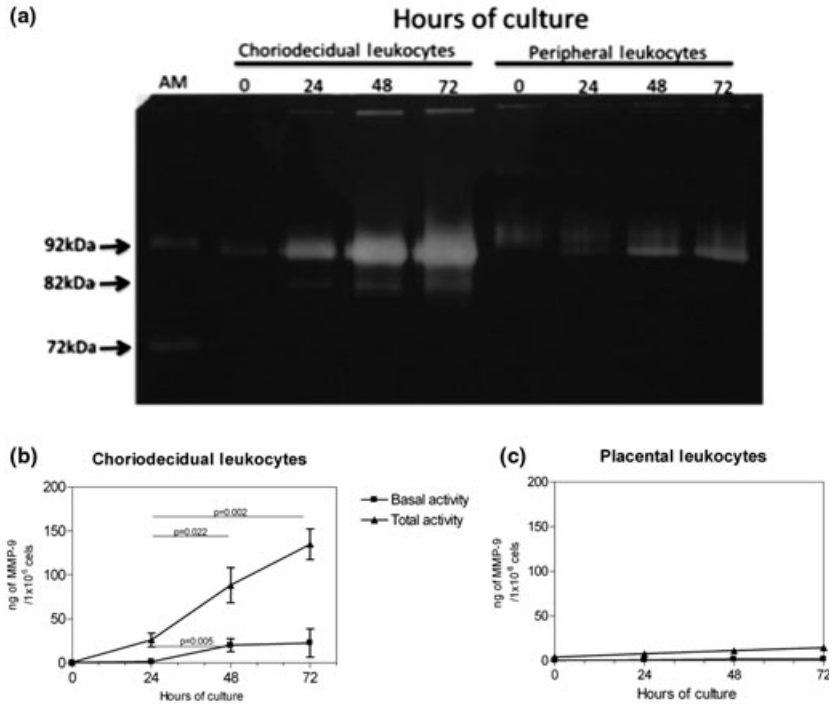


**Fig. 2** MMP-9 activity by zymography and ELISA. (a) Zimography. The culture media of choriodecidual and intervillous placental blood leukocytes ($n$ = 7) collected at 0, 24, 48, and 72 h were analyzed in gelatin-substrate gels. The electrophoretic positions of the gelatinolytic activities (clear bands) show the 92 kDa inactive enzyme and the 82 kDa active form of MMP-9, respectively. AM = culture medium of U937 cells as activity marker. (b) ELISA. Active and total MMP-9 in the same media was quantitated. Each point represents the mean ± S.D. ($n$ = 3).

IL-6.[4,5,9,12] Increased concentrations of these cytokines have been documented during labor in different compartments, including umbilical cord blood, amniotic fluid, and peripheral maternal blood.[3,11,16,38] Choriodecidual cells may be a major source for these signals. These cytokines have been proposed as a first wave of signaling, acting on local cells and resulting in the production of a secondary wave of effector molecules.[7,10,18,21] Even though we cannot totally integrate the resulting molecular crosstalk between leukocytes and the local cells in the choriodecidua, supporting experimental data allow us to suggest that cytokines secreted by choriodecidual leukocytes may explain secretion and activation of MMP-9 under the conditions in our experiments. Choriodecidual leukocytes may produce three times more MMP-9 than reference cell lines such as U937[14] or amounts equivalent to those produced by some metastatic cancer lines. In addition to the above-mentioned choriodecidual leukocyte functional properties, our data support the possibility that these cells could be contributing to the secondary wave of mediators, creating a microenvironment leading to collagenolysis, which could be related to the rupture of the fetal membranes.[10,18]

In summary, our findings demonstrate that choriodecidual leukocytes isolated from fetal membranes at term are functionally different from cells in other compartments and may collaborate to modulate the microenvironment linked to induction and progression of human labor.

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# Evidence for a Role for the Adaptive Immune Response in Human Term Parturition

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## 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.

## 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 molecules (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 represents '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 and 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 consent 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 1× 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× 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× 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× 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 $cm^2$ pieces and washed gently in 1× 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× 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 CD45RA-PE 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× 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 Zeiss, 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. Reverse 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 $\leq 0.05$ 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. 1b).

### Choriodecidual Leukocytes Include $CD4^+$ T Cells at Term Pregnancy and Also $CD4^-CD8^-$ T Cells During Spontaneous 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 ($P < 0.0001$ each). Importantly, the proportion of DN T cells were higher in the choriodecidua obtained from patients who had undergone spontaneous labor

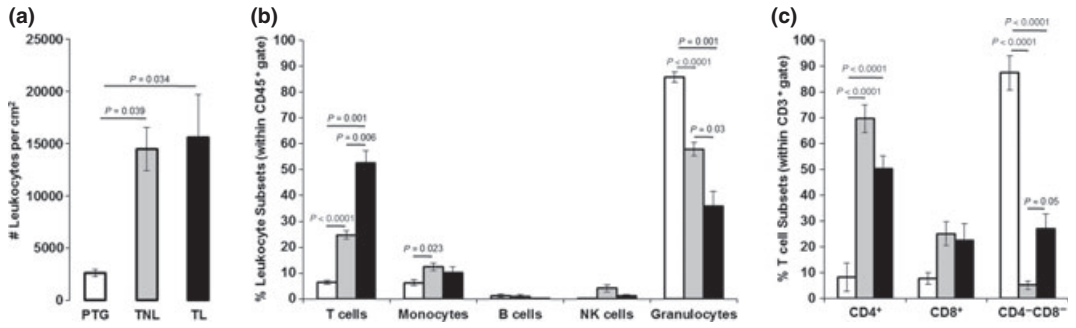


**Fig. 1** Number and phenotype of choriodecidual leukocytes. PTG, white; TNL, gray; TL, black bars. (a) Number of leukocytes per $cm^2$ of tissue. Leukocyte density increased at term of pregnancy. (b) Leukocyte subsets were analyzed within the $CD45^+$ gate. While T cells increased, granulocytes decreased 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 than in TNL. Data shown are means ± S.E.M., with five to seven tissues per group.

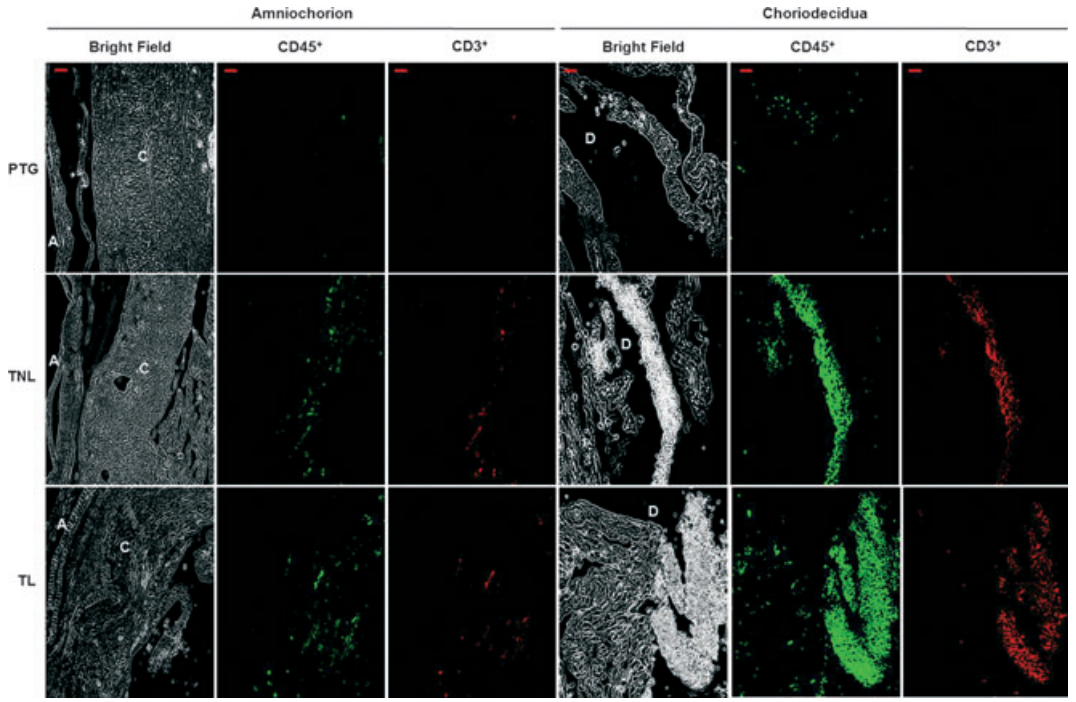


**Fig. 2** Tissue localization of choriodecidual leukocytes. Photomicrograph of amniochorion (left panel) and choriodecidua (right panel) in each group. A, amnion; C, chorion; D, decidua. Leukocytes ($CD45^+$) were greater in choriodecidua than in amniochorion, and their density seemed to increase from 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 ×200. Data are representative of three or more independent experiments with five tissues per group.

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

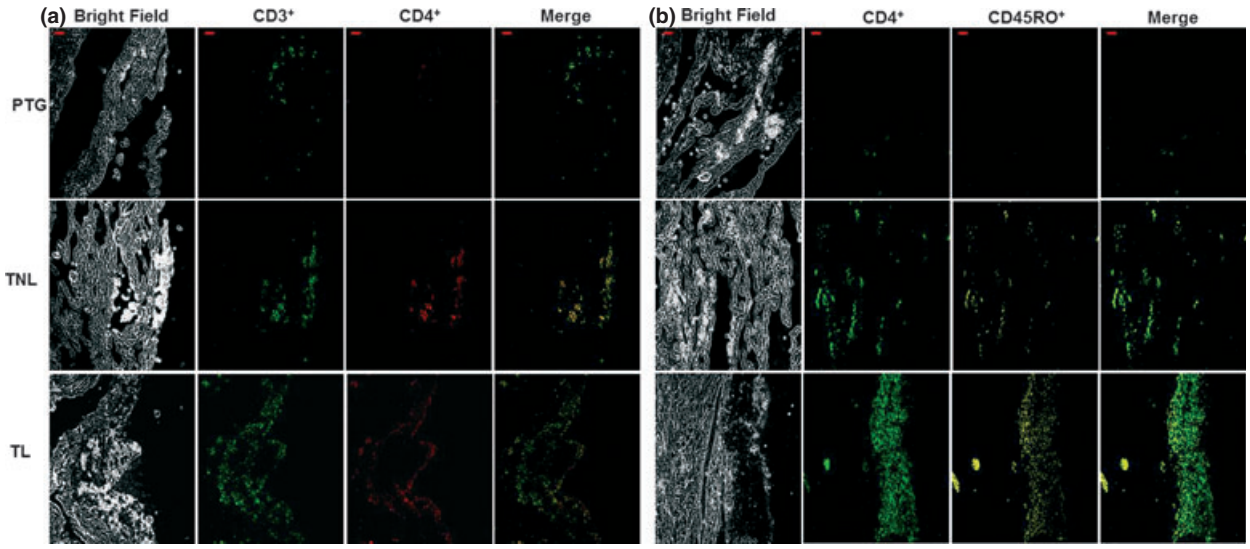


**Fig. 3** Tissue localization of CD45RO$^+$ memory-like T cells. (a) Photomicrograph of choriodecidua identifying CD4$^+$ T cells in each group. (b) Photomicrograph of choriodecidua identifying CD4$^+$ CD45RO$^+$ memory-like T cells in each group. Merge shows the co-localization of both markers. CD45RO$^+$ memory-like T cells seemed to increase from PTG to TL. Bar, 20 μm. Confocal microscopy: magnification ×200. Data are representative of three or more independent experiments with five tissues per group.

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 CD45RO$^+$ '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 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),

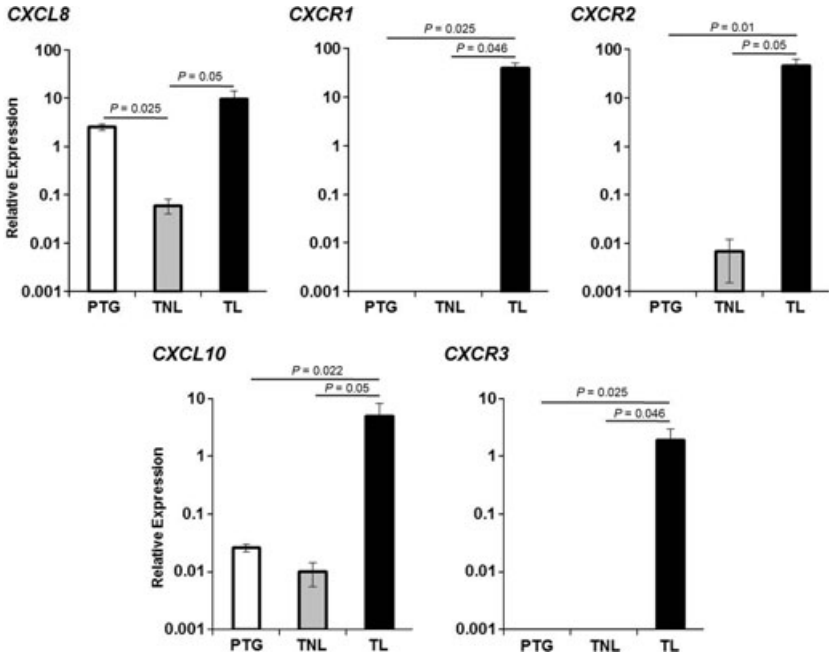


**Fig. 4** Chemokine expression. Relative expression of *CXCL8* and *CXCL10* and its receptors *CXCR1-3* in choriodecidual leukocytes from each group. *CXCL8* was higher in TL than in TNL, and lower in TNL than in PTG. *CXCL10*, *CXCR1*, *CXCR2* and *CXCR3* were higher in TL than in TNL and PTG. Data are expressed as relative expression using *ACTB* gene as the reference. Data shown are means ± S.E.M., with five to seven tissues per group.

*ICAM2*, *ICAM3*, *VCAM* (vascular cell adhesion molecule), *SELP* (selectin 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 TNF-α, IL-1β and MMP-9 During Spontaneous Labor at Term

Finally, the expression of TNF alpha 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 from women who had experienced labor in term gestation (Fig. 7).

## Discussion

### Principal Findings of the Study

(i) Memory-like $CD4^+$ T cells are present in the choriodecidual tissues of women at term without labor,

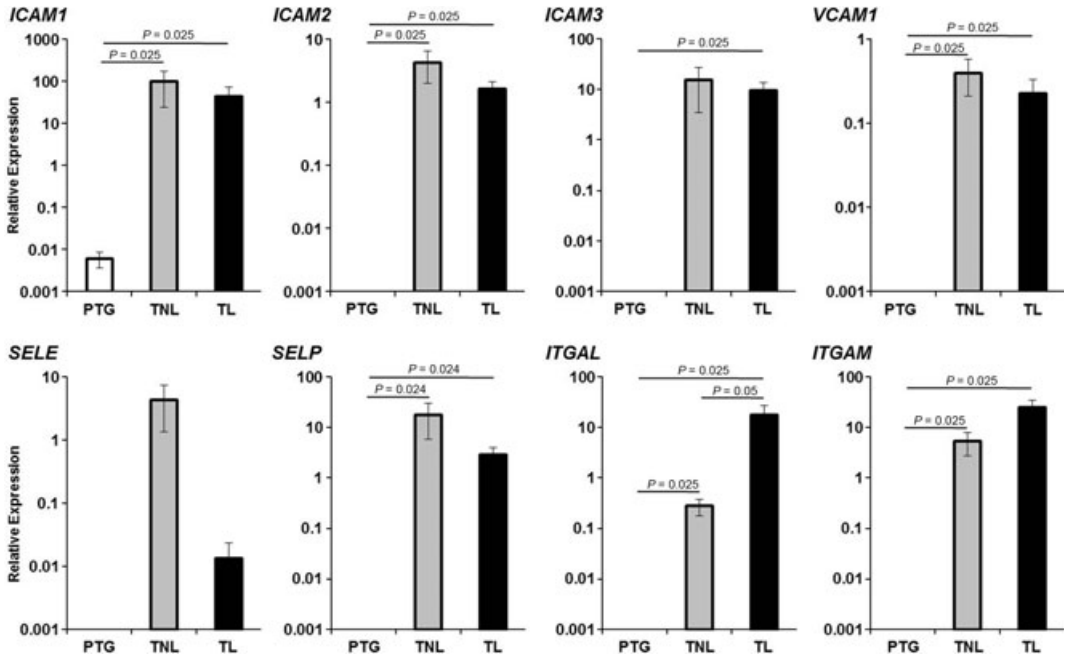


**Fig. 5** Cell adhesion molecules expression. Relative expression of *ICAM1*, *ICAM2*, *ICAM3*, *VCAM*, *SELP*, *SELE*, *ITGAL* and *ITGAM* in choriodecidual leukocytes from each group. All of them, except *SELE*, were higher in TL than in PTG. *ICAM1*, *ICAM2*, *VCAM*, *SELP*, *ITGAL* and *ITGAM* were 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 ± S.E.M., with five to seven tissues per group.

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, IL-1β, 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 the 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] It is important to note that inflammation (indicated by leukocyte infiltration and cytokine expression) occurs at the time of peri-implantation and therefore, it is important for successful reproduction.[43,55,118–129] This process is

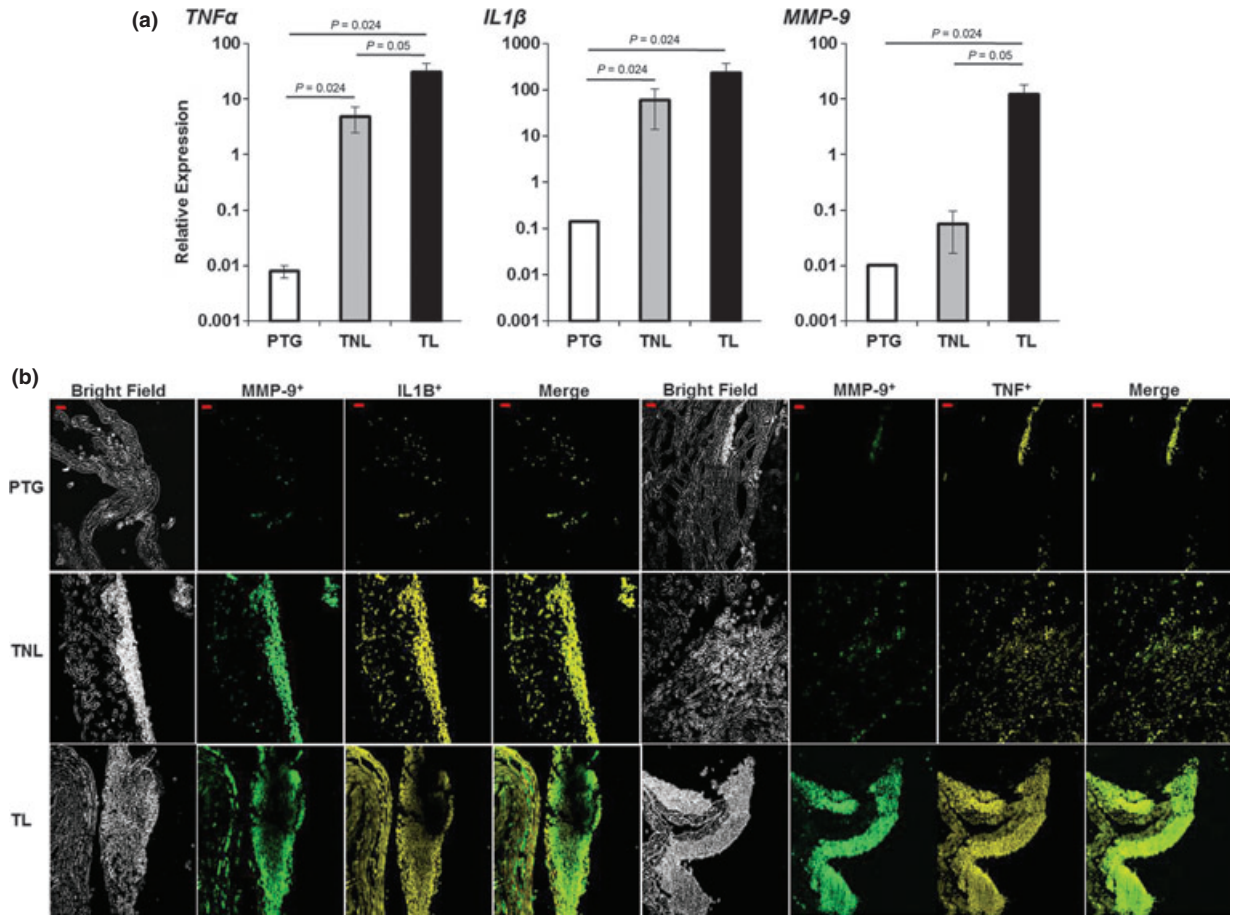


**Fig. 6** Labor mediators. (a) Expression of labor mediators. *IL-1β*, *TNF-α*, and *MMP9* in choriodecidual leukocytes from each group. All three mediators increased from PTG to TL. Data are expressed as relative expression using *ACTB* gene as the reference. Data shown are means ± S.E.M., with five 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 ×200. Data are representative of three or more independent experiments with five tissues per group.

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.[4,74,80] 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

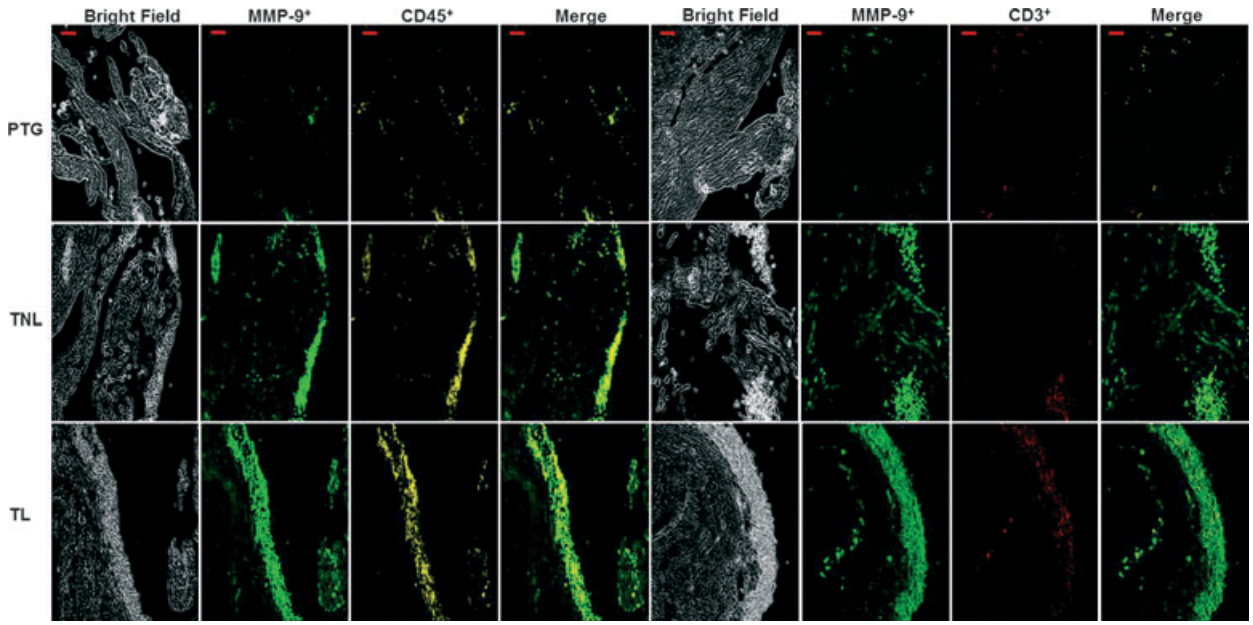


**Fig. 7** Localization of MMP-9 in choriodecidual leukocytes. Photomicrograph of choriodecidua identifying MMP-9$^+$CD45$^+$ cells or MMP-9$^+$CD3$^+$ T cells in each group. Both MMP-9$^+$ total leukocytes and T cells increased from PTG to TL. Merge shows the co-localization of both markers. Bar, 20 μm. Confocal microscopy: magnification ×200. Data are representative of three or more independent experiments with five tissues per group.

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 Spontaneous Labor at Term

T 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 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 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 its 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 choriodecidual 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 an 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 Implicated 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 its receptors, *CXCR1* and *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. Alternatively 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 MMP9 in the choriodecidua during spontaneous labor at term pregnancy. We

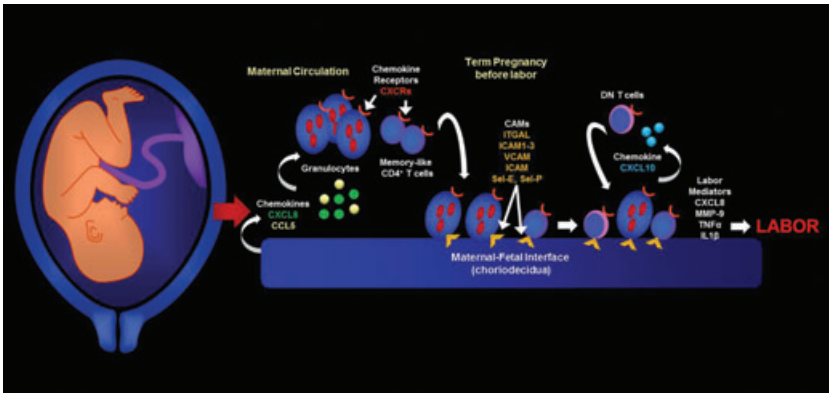


**Fig. 8** Conceptual framework. Choriodecidual tissues express chemokines to recruit maternal circulating leukocytes at term pregnancy. Before the onset of labor, choriodecidual memory-like $CD4^+$ T cells and other leukocytes such as granulocytes express cell adhesion molecules to remain into this 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.

recognized the following limitations: (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 the 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.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** (A) Representative diagram of spread out fetal membranes. Area was calculated by measuring the dotted lines. (B) Representative dot-plots showing the dual parameters used for the flow cytometric analysis. Monocytes ($CD45^+CD14^+$), NK cells ($CD45^+CD56^+$), B cells ($CD45^+CD19^+$) and T cells ($CD45^+CD3^+$) were analyzed within the $CD45^+$ gate. T cell subsets were analyzed within the $CD3^+$ gate. P4 – $CD3^+CD8^+$, P5 – $CD3^+CD4^+$, P6 – $CD3^+CD4^-CD8^-$.
**Table S1.** Panel of antibodies used in this study.
**Table S2.** Primers and probes used for performing real time PCR.