Immunobiology 224 (2019) 177–181

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Short communication

A trypsin-based method for isolating leukocytes from human choriodecidua suitable for immunophenotyping and transcriptome studies

Karla MacDonald-Ramos[a], Marcia Arenas-Hernandez[a,d], Ismael Mancilla-Herrera[b], Claudia Rangel-Escareño[c], Rodrigo Vega-Sanchez[a,*]

[a] Departamento de Nutrición y Bioprogramación, Instituto Nacional de Perinatología Isidro Espinosa de los Reyes, Montes Urales 800, Lomas Virreyes, 11000 Mexico City, Mexico
[b] Departamento de Infectología e Inmunología, Instituto Nacional de Perinatología Isidro Espinosa de los Reyes, Montes Urales 800, Lomas Virreyes, 11000 Mexico City, Mexico
[c] Departamento de Genómica Computacional, Instituto Nacional de Medicina Genómica, Periférico Sur 4809, Arenal Tepepan, 14610 Mexico City, Mexico
[d] Departamento de Biomedicina Molecular Centro de Investigación y de Estudios Avanzados, Instituto Politécnico Nacional Av Instituto Politécnico Nacional 2508, La Laguna Ticoman, 07360 Mexico City, Mexico

ARTICLE INFO

Keywords:
Choriodecidua
Leukocytes
Isolation
Trypsin
Flow cytometry
qRT-PCR
Microarrays

ABSTRACT

Leukocytes found at the human maternal-fetal interface participate in the inflammatory process associated with both preterm and term labor; therein, effective methods for their isolation that allow further phenotypic and functional analyses are necessary. Leukocyte isolation is usually carried out through scraping or enzyme digestion of the choriodecidua, however both methods usually limit the use of downstream immunophenotyping or transcriptomic techniques. Here we describe an isolation method based on gentle trypsin digestion that yields a leukocyte-enriched cell mixture with high lymphocyte viability, although less viable myeloid cells. We show that the method does not compromise cell surface markers since isolated leukocytes are suitable for flow cytometry; and that high quality RNA can be obtained from these cells for qRT-PCR and microarray analyses.

1. Introduction

Leukocytes that infiltrate the maternal-fetal interface seem to play an important role in the local inflammatory process established during both preterm and term human labor (Gomez-Lopez et al., 2014). Maternal leukocytes, both innate and adaptive, are recruited to the choriodecidual portion of the maternal-fetal interface through selective chemotaxis (Gomez-Lopez et al., 2013a, 2009). Once infiltrated, they contribute to establish a local pro-inflammatory environment by secreting several effector molecules including cytokines, chemokines, prostaglandins and metalloproteinases (Challis et al., 2009; Norman et al., 2007). Therefore, effective methods for isolating these cells that allow further phenotypic and functional analyses are necessary.

Commonly used isolation methods include scraping of the choriodecidua (Nagaeva et al., 2002), enzymatic digestion (White et al., 2000) or their combination (Xu et al., 2015). Scraping is suitable for flow cytometry (Gomez-Lopez et al., 2013b), but yields fewer cells with compromised viability (Ritson and Bulmer, 1987) and diminished nucleic acid integrity. Enzyme-based isolation yields higher leukocyte numbers while preserving cellular and molecular integrity, but may lead to loss of cell surface antigens particularly when using proteases for long incubation periods (e.g. 2 h) (White et al., 2000), thereby reducing the possibility of analysis through immunophenotyping.

Here we describe a method for isolating human choriodecidual leukocytes combining gentle trypsin digestion with magnetic cell sorting, and show its compatibility with downstream flow cytometry, qPCR and microarray analyses.

2. Methods

2.1. Biological samples

We obtained fetal membranes from women with term pregnancies (≥37 weeks of gestation), without infection or autoimmune pathologies (Table 1), that delivered at the Instituto Nacional de Perinatología in Mexico City. All participants signed an informed consent (project

* Corresponding author at: Departamento de Nutrición y Bioprogramación, Instituto Nacional de Perinatología "Isidro Espinosa de los Reyes", Montes Urales 800, Lomas Virreyes, 11000 Ciudad de México, Mexico.
E-mail addresses: kmmacdonald@yahoo.com (K. MacDonald-Ramos), march.arenas@gmail.com (M. Arenas-Hernandez), mahi_25803@yahoo.com.mx (I. Mancilla-Herrera), crangel@inmegen.gob.mx (C. Rangel-Escareño), vegarodrig@gmail.com (R. Vega-Sanchez).

https://doi.org/10.1016/j.imbio.2018.09.001
Received 19 June 2018; Received in revised form 4 September 2018; Accepted 19 September 2018
Available online 22 September 2018

Table 1
Characteristics of participating women.

	Flow cytometry samples (n = 9)	RNA samples (n = 15)	qRT-PCR samples (n = 6)	Microarray samples (n = 10)
Maternal age	31 (16–40)	29 (16–35)	25 (18–41)	27.5 (16–35)
Gestational age (weeks)	39.3 (33.0–40.2)	38.5 (36.6–40.6)	38.8 (38.1–40.2)	38.5 (36.6–40.0)
Number of pregnancies	2.0 (1–5)	2.0 (1–5)	1.5 (1–4)	2.0 (1–3)
Newborn's sex	Female = 5 Male = 4	Female = 6 Male = 9	Female = 5 Male = 1	Female = 4 Male = 6
Labor / no labor	2/7	8/7	0/6	5/5
Route of delivery (vaginal / c-section)	2/7	7/8	0/6	5/5

Values are presented as medians (range). Most women delivered by elective cesarean section due to personal or familial history of pregnancy-related complications according to institutional policies.

register 212250-02191). Membranes were cut from the placenta, placed in sterile containers and transported to the laboratory within the first hour.

2.2. Isolation of choriodecidual leukocytes

We manually separated chorion from amnion (which was discarded), removed blood clots, and cut the chorion together with decidua parietalis into small pieces (approximately 3 × 3 cm). We then incubated the pieces at 37 °C for 10–15 min with gentle shaking at approximately 80 × RPM in 100 ml of DMEM containing 0.1% trypsin (100 mg/ml).

Other reported isolation techniques use trypsin as part of a protease cocktail with long incubation periods. White et al. used 1.4 mg/ml of trypsin together with pancreatin, dispase, collagenase and hyaluronidase, and incubated for 2 h (White et al., 2000). Xu et al. used mechanical disaggregation of the tissue followed by a 45 min incubation period in a commercial cocktail containing unspecified amounts of proteolytic and collagenolytic enzymes (Xu et al., 2015). In our technique, although we used trypsin at a much higher concentration (100 mg/ml, corresponding to 0.1%), we did not add any other proteases and the incubation time was only 10–15 min. As we will show, this allowed for an efficient recovery of choriodecidual leukocytes while preserving the integrity of both the cells and their surface antigens.

Following enzyme digestion, the medium was filtered with a gauze, diluted 1:1 with 1X PBS and cells were pelleted by centrifugation (2500 rpm–10 min–4 °C). Cells were then incubated in erythrocyte lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) for 20 min with low agitation, washed with 1X PBS and filtered through a 30 µm pre-separation filter (Miltenyi Biotec, USA).

Afterwards, we performed magnetic cell sorting (MACS): cells were resuspended in 500 µl of PBE buffer (1X PBS, 0.5% bovine serum albumin, 2 mM EDTA), incubated with an anti-human CD45 microbead-conjugated antibody, sorted through positive selection columns (Miltenyi Biotec, USA) and counted in a Beckman Coulter AcT-5diff hematology analyzer. Bound antibody was not dissociated from cells after sorting. All buffers were ice-cooled to minimize RNA degradation.

2.3. Leukocyte identification and viability by flow cytometry

To analyze the leukocyte yield of the isolation technique and its adequacy for downstream flow cytometry, we immunostained six different cell samples (n = 6) before and after MACS. We incubated cells in 100 µl PBE with titrated volumes of the following anti-human fluorochrome-conjugated monoclonal antibodies: CD45/PE-Cy7, CD4/APC-Cy7, CD56/Brilliant Violet 421 (Biolegend, San Diego, CA, USA); CD3/Brilliant Violet 510, CD19/FITC, CD14/PE (BD Biosciences, San Jose, CA, USA), CD8/APC (Invitrogen, Frederick, MD, USA), CD15/PC5 (Beckman Coulter, Indianapolis, IN, USA). After staining, we fixed cells with 1x FACS Lysing solution (Cat 349202, BD Biosciences, USA).

To analyze leukocyte viability, we incubated cells with anti-CD45/Pacific Blue (BioLegend, San Diego, CA, USA) and Fixable Viability Dye eFluor506 (FVD, eBiosciences, Carlsbad, CA, USA), and then fixed them as described above. We analyzed cells in a FACS Aria III flow cytometer using the DIVA software V.6.1.3 (BD Biosciences, USA), counting at least 10,000 events.

For leukocyte identification, we gated singlet events from a 45-degree diagonal line by FSC-A vs FSC-H dot plot; lymphocytes were selected as low complexity (SSC) events expressing high levels of CD45, while myeloid cells were defined as heterogeneous complexity and medium expression of CD45. From lymphocytes, we defined helper T cells as CD3 + CD4 +, cytotoxic T cells as CD3 + CD8 +, B cells as CD19 +, and natural killer (NK) as CD56 + cells. From myeloid cells, we selected neutrophils as CD15 + and monocytes as CD14 + cells (Fig. 1A). For viability analysis, we identified live cells (FVD-) in lymphoid and myeloid cells gated according their complexity and CD45 expression (Fig. 1C).

2.4. RNA quantification, integrity and cDNA synthesis

We obtained total RNA using Trizol reagent (Invitrogen, USA) and removed any residual DNA following the DNAse I treatment of RNAqueous-4PCR kit (Ambion, USA). We then quantified it in a Nanodrop 2000 spectrophotometer and obtained the RNA integrity number (RIN) with an Agilent Bioanalyzer 2100 using RNA 6000 Nano kit (Thermo Scientific, USA).

For qRT-PCR, we synthesized cDNA from 100 ng of total RNA using the RT^2 First Strand Kit (Qiagen Sciences, USA) in a GeneAmp 9700 PCR System (Applied Biosystems, Singapore). For microarrays, 200 ng of total RNA were amplified with an Ambion WT Expression kit (Ambion, USA), then cDNA was fragmented and labeled with a GeneChip® WT Terminal Labeling kit (Affymetrix, USA).

2.5. Gene expression by real-time qRT-PCR

We analyzed the expression of 211 inflammation-related genes and 5 reference genes in six different samples (not pooled), using RT^2 Profiler PCR Arrays for Common Cytokines, Chemokines and Receptors, and Extracellular Matrix & Adhesion Molecules together with the RT^2 SYBR Green ROX qPCR Mastermix (Qiagen Sciences, USA). Arrays were analyzed in a Viia 7 Real-Time PCR System (Applied Biosystems, Singapore) with one cycle at 95 °C–10 min and 40 amplification cycles (95 °C–15 s, 60 °C–1 min, 95 °C–15 s).

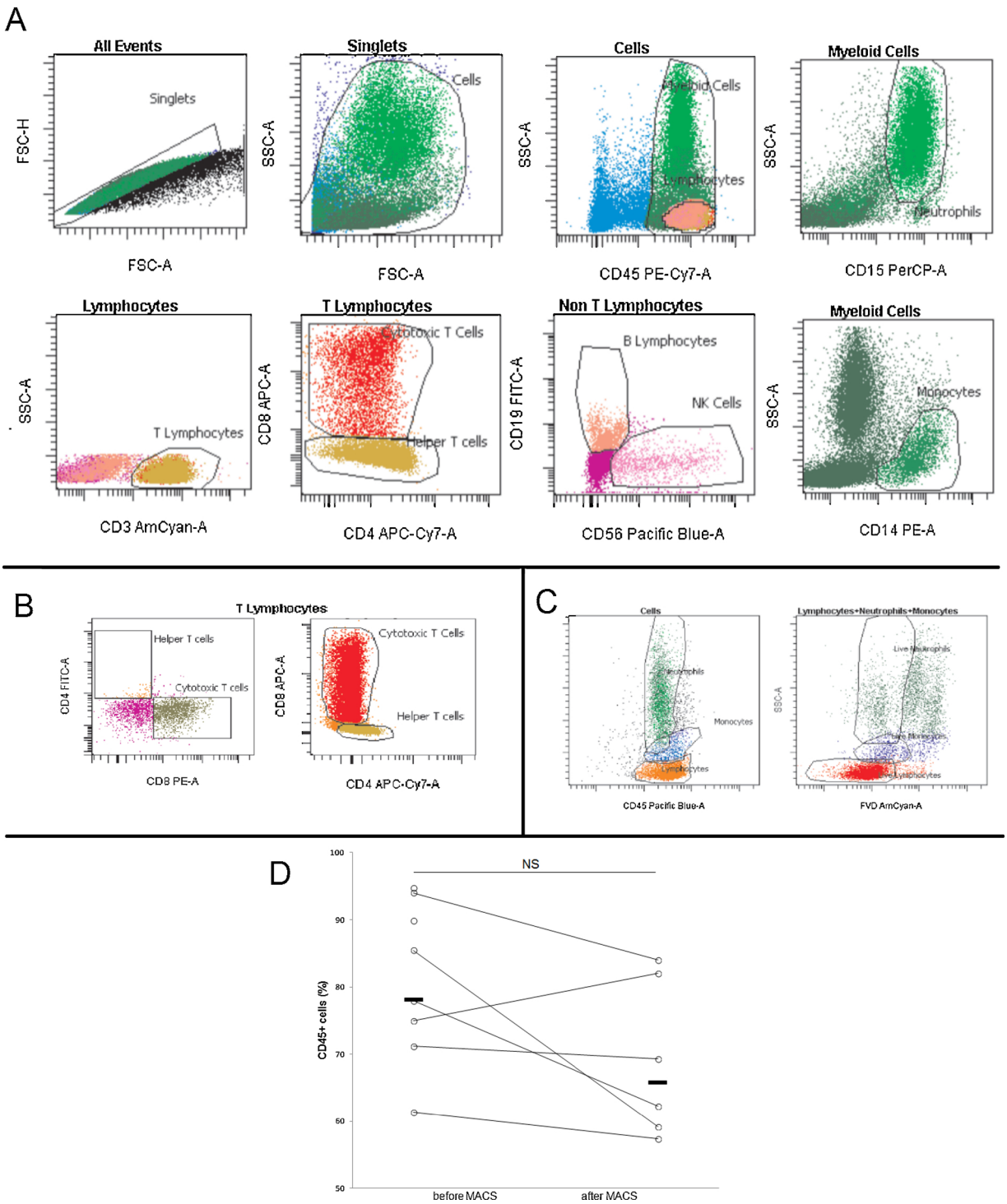

Fig. 1. Flow cytometry immunophenotyping and viability analysis of isolated choriodecidual leukocytes.
A: Immunophenotyping of leukocyte subpopulations. **B**: Comparison of FITC vs APC-Cy7 for identifying CD4 + T cells. **C**: Lymphocyte, monocyte and neutrophil viability after isolation. **D**: Proportion of CD45+ cells before and after magnetic cell sorting. Small black lines represent medians. NS = no significant difference between groups using Mann-Whitney test ($p = 0.088$). Image shows representative data of three (sections A, B and C) or six independent experiments (section D).

2.6. Transcriptome analysis by microarrays

We analyzed the transcriptome of choriodecidual leukocytes in ten individual samples (not pooled) with RIN values ≥7 (min. 7.0–max. 9.6), using GeneChip Human Gene 1.0 ST arrays (Affymetrix, USA). Raw data was background-corrected using the Robust Multi-array Average Method and normalized using the Quantile Normalization Method (Irizarry et al., 2003).

3. Results and discussion

With the isolation method here described, a leukocyte-enriched choriodecidual cell mixture can be obtained, which is suitable for both immunophenotyping and transcriptome analyses. We obtained a median amount of 3.25×10^6 isolated cells (min 0.17×10^6–max 25.8×10^6 cells, n = 41) comprising a median of 78% CD45+ leukocytes (min. 61%–max. 94%). This variability in cell purity was

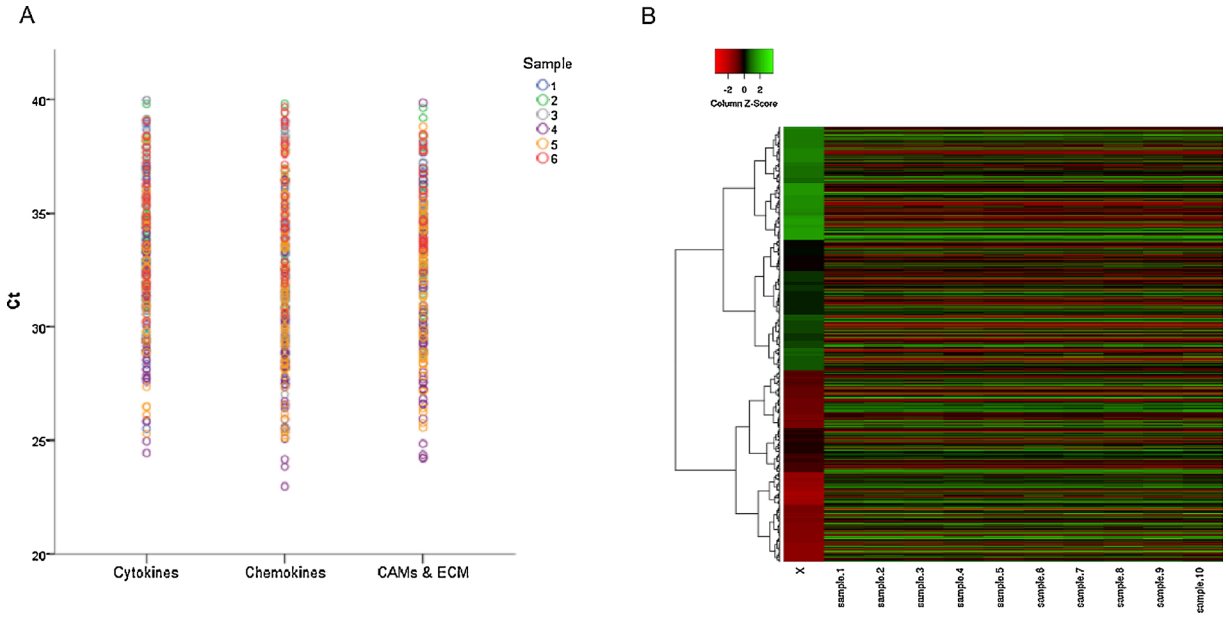

Fig. 2. Downstream expression studies in isolated leukocytes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).
A: Graph shows Ct values of each individual gene (circles) within the cytokines, chemokines and CAMS-ECM qRT-PCR arrays (n = 6); reactions presenting more than one peak on the melting curve or without an amplification signal (one third of the reactions, on average) were excluded. **B:** Heat map shows the unsupervised hierarchical clustering of 600 genes randomly selected from the 2501 expressed genes (n = 10). Negative z-scores indicate lower expression (shown in red) while the positive z-scores indicate higher expression (shown in green). The heatmap was constructed using the freely available online tool Heatmapper (www.heatmapper.ca).

associated with the individual researcher's experience with the isolation technique.

Previously reported methods have shown that tissue digestion with enzyme cocktails for long periods (i.e. 2 h) results in the loss of several cell surface markers (White et al., 2000). The gentle trypsin digestion used in our method did not compromise the most common leukocyte markers since immunophenotyping by flow cytometry was successful (Fig. 1A), which represents a major advantage of this technique. While this was true for most leukocyte markers (CD45, CD3, CD8 CD56, CD15 and CD14), CD4 and CD19 expression, in helper T and B cells respectively, was notably lower than expected; CD4 + cells, for example, were only detectable using a high-energy fluorochrome such as APC-Cy7, but not FITC (Fig. 1B). This may be due to the susceptibility to proteolytic activity of trypsin over the CD4 molecule (Harris et al., 1990), which has also been observed with other enzyme-based isolation procedures (Autengruber et al., 2012).

Functional assays usually require that isolated cells have high viability. Our leukocyte isolation procedure yielded high percentages of live lymphocytes (median 90%) but decreased frequencies of live monocytes (median 50%) and neutrophils (median 27%). Isolated cells would be suitable for lymphocyte cultures, which can survive for longer periods of time, but would be limited for myeloid cell cultures (Fig. 1C).

Contrary to our expectations, MACS enrichment did not increase overall leukocyte frequencies but tended to decrease them, although not significantly (Fig. 1D). This was also observed by Tilburgs et al. when trying to purify lymphocytes from decidual cell mixtures (Tilburgs et al., 2008). The lower CD45 signal observed after cell sorting may be due to some MACS antibody still being coupled to the CD45 molecule (since this antibody was not removed after sorting) thereby limiting the subsequent coupling of the staining antibody.

High quality RNA, suitable for qRT-PCR and microarray analyses, may be obtained from leukocytes isolated through this method. Total RNA had a median concentration of 240.82 ng/μl (min 26.36 ng/μl–max 1816.65 ng/μl), a median 260/280 ratio of 1.89 and a median RIN of 7.8 (min 5.7–max 9.6). All samples analyzed through qRT-PCR yielded expression values of more than half of the analyzed target genes and all reference genes, with Ct values ranging from 22.97 to 39.98. Microarrays showed 2501 genes expressed by choriodecidual cells (Fig. 2). Although using samples with the highest possible RIN values (above 9.0) for microarray analyses is a common practice, our microarray results showed that samples with RIN values between 7 and 9 also yield good expression profiles and may therefore be adequate for such analyses.

Finally, by coupling the method described here together with purifying techniques such as fluorescence-activated cell sorting (FACS), other components of the maternal-fetal interface, such as decidual cells or even amniocytes, may be isolated, as these also play an important role in the inflammatory processes at the maternal-fetal interface (El-Azzamy et al., 2017; Flores-Espinosa et al., 2014).

4. Conclusion

We describe a method for isolating choriodecidual leukocytes that yields an enriched cell mixture with high lymphocyte viability although less viable myeloid cells. Isolated leukocytes are suitable for downstream flow cytometry and high quality RNA can be obtained for qRT-PCR and microarray analyses. The isolation method here described could help answering questions regarding the role of specific leukocyte subpopulations in the inflammatory process that characterizes human labor; the genetic and epigenetic mechanisms involved in such process; the establishment and loss of immune tolerance at the maternal-fetal interface; among many others.

Conflict of interest

Authors declare not having any financial, personal or commercial conflict of interest.

Acknowledgements

We are grateful to Raul Mojica-Espinosa for his assistance on microarray experiments. This work was supported by the Consejo

Nacional de Ciencia y Tecnología, México (CONACYT, grant SALUD-2010-1-141144) and by the Instituto Nacional de Perinatología, México (project 212250-02191). Authors declare having no conflicting interests.

References

Autengruber, A., Gereke, M., Hansen, G., Hennig, C., Bruder, D., 2012. Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. Eur. J. Microbiol. Immunol. 2, 112–120. https://doi.org/10.1556/EuJMI.2.2012.2.3.

Challis, J.R., Lockwood, C.J., Myatt, L., Norman, J.E., Strauss 3rd, J.F., Petraglia, F., 2009. Inflammation and pregnancy. Reprod. Sci. 16, 206–215.

El-Azzamy, H., Balogh, A., Romero, R., Xu, Y., LaJeunesse, C., Plazyo, O., Xu, Z., Price, T.G., Dong, Z., Tarca, A.L., Papp, Z., Hassan, S.S., Chaiworapongsa, T., Kim, C.J., Gomez-Lopez, N., Than, N.G., 2017. Characteristic changes in decidual gene expression signature in spontaneous term parturition. J. Pathol. Transl. Med. 51, 264–283. https://doi.org/10.4132/jptm.2016.12.20.

Flores-Espinosa, P., Pineda-Torres, M., Vega-Sánchez, R., Estrada-Gutiérrez, G., Espejel-Nuñez, A., Flores-Pliego, A., Maida-Claros, R., Paredes-Vivas, Y., Morales-Méndez, I., Sosa-González, I., Chávez-Mendoza, A., Zaga-Clavellina, V., 2014. Progesterone elicits an inhibitory effect upon LPS-induced innate immune response in pre-labor human amniotic epithelium. Am. J. Reprod. Immunol. 71, 61–72.

Gomez-Lopez, N., Estrada-Gutierrez, G., Jimenez-Zamudio, L., Vega-Sanchez, R., Vadillo-Ortega, F., 2009. Fetal membranes exhibit selective leukocyte chemotaxic activity during human labor. J. Reprod. Immunol. 80, 122–131.

Gomez-Lopez, N., Tanaka, S., Zaeem, Z., Metz, G.A., Olson, D.M., 2013a. Maternal circulating leukocytes display early chemotactic responsiveness during late gestation. BMC Pregnancy Childbirth 13 (Suppl. 1), S8. https://doi.org/10.1186/1471-2393-13-S1-S8 1471-2393-13-S1-S8 [pii].

Gomez-Lopez, N., Vega-Sanchez, R., Castillo-Castrejon, M., Romero, R., Cubeiro-Arreola, K., Vadillo-Ortega, F., 2013b. Evidence for a role for the adaptive immune response in human term parturition. Am. J. Reprod. Immunol. 69, 212–230. https://doi.org/10.1111/aji.12074.

Gomez-Lopez, N., StLouis, D., Lehr, M.A., Sanchez-Rodriguez, E.N., Arenas-Hernandez, M., 2014. Immune cells in term and preterm labor. Cell. Mol. Immunol. 11, 571–581. https://doi.org/10.1038/cmi.2014.46 cmi201446 [pii].

Harris, R.J., Chamow, S.M., Gregory, T.J., Spellman, M.W., 1990. Characterization of a soluble form of human CD4: peptide analyses confirm the expected amino acid sequence, identify glycosylation sites and demonstrate the presence of three disulfide bonds. Eur. J. Biochem. 188, 291–300. https://doi.org/10.1111/j.1432-1033.1990.tb15402.x.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., Speed, T.P., 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264. https://doi.org/10.1093/biostatistics/4.2.2494/2/249 [pii].

Nagaeva, O., Bondestam, K., Olofsson, J., Damber, M.-G., Mincheva-Nilsson, L., 2002. An optimized technique for separation of human decidual leukocytes for cellular and molecular analyses. Am. J. Reprod. Immunol. 47, 203–212. https://doi.org/10.1034/j.1600-0897.2002.01065.x.

Norman, J.E., Bollapragada, S., Yuan, M., Nelson, S.M., 2007. Inflammatory pathways in the mechanism of parturition. BMC Pregnancy Childbirth 7 (Suppl. 1), S7.

Ritson, A., Bulmer, J.N., 1987. Extraction of leucocytes from human decidua. A comparison of dispersal techniques. J. Immunol. Methods 104, 231–236. https://doi.org/10.1016/0022-1759(87)90509-6.

Tilburgs, T., Repnik, U., van der Mast, B.J., de Groot-Swings, G.M., Roelen, D.L., Claas, F.H., Scherjon, S.A., 2008. Do's and don'ts in isolation and purification of decidual lymphocytes and macrophages. In: Tilburgs, T. (Ed.), Fetus Specific Immune Recognition and Regulation by T Cells at the Fetal-Maternal Interface in Human Pregnancy. T. Tilburgs, Leiden, pp. 139 ISBN 978-90-9023597-4.

White, H.D., Prabhala, R.H., Humphrey, S.L., Crassi, K.M., Richardson, J.M., Wira, C.R., 2000. A Method for the Dispersal and Characterization of Leukocytes From the Human Female Reproductive Tract. AJRI.

Xu, Y., Plazyo, O., Romero, R., Hassan, S.S., Gomez-Lopez, N., 2015. Isolation of leukocytes from the human maternal-fetal interface. J. Vis. Exp., e52863. https://doi.org/10.3791/52863.