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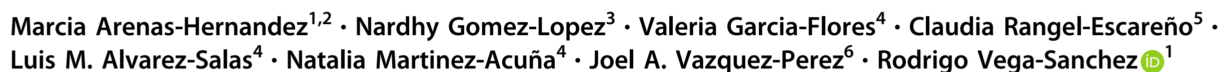
ARTICLE

Choriodecidual leukocytes display a unique gene expression signature in spontaneous labor at term



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Abstract

Prior to and during the process of human labor, maternal circulating leukocytes infiltrate the maternal-fetal interface (choriodecidua) and become activated resembling choriodecidual leukocytes. Since, there is no evidence comparing maternal circulating and choriodecidual leukocytes, herein, we characterized their transcriptome and explored the biological processes enriched in choriodecidual leukocytes. From women undergoing spontaneous term labor we isolated circulating and choriodecidual leukocytes, performed microarray analysis ($n = 5$) and qRT-PCR validation ($n = 9$) and interaction network analysis with up-regulated genes. We found 270 genes up-regulated and only 17 genes down-regulated in choriodecidual leukocytes compared to maternal circulating leukocytes. The most up-regulated genes were *CCL18, GPNMB, SEPP1, FN1, RNASE1, SPP1, C1QC*, and *PLTP*. The biological processes enriched in choriodecidual leukocytes were cell migration and regulation of immune response, chemotaxis, and humoral immune responses. Our results show striking differences between the transcriptome of choriodecidual and maternal circulating leukocytes. Choriodecidual leukocytes are enriched in immune mediators implicated in the spontaneous process of labor at term.

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41435-017-0010-z) contains supplementary material, which is available to authorized users.

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Introduction

Labor is recognized as a physiological process of sterile inflammation [1–5], characterized by the infiltration of immune cells into the cervix [6–9], myometrium [8, 10–12], and maternal-fetal interface [8, 13–16]. This interface includes two areas of contact between the mother and the fetus: the decidua parietalis, which lines the uterine cavity not covered by the placenta and is juxtaposed to the chorion laeve; and the decidua basalis, which lays on the basal plate of the placenta and is invaded by the interstitial trophoblast [16, 17]. Decidua parietalis is composed of stroma-type cells, glandular cells, and leukocytes [18–21], and the portion that is attainable is attached to the chorioamniotic membranes; therefore, this anatomical site is known as the choriodecidua [16]. Since the choriodecidua represents the major site of fetal antigenic exposure to the maternal immune system, our studies have focused on understanding the mechanisms whereby maternal leukocytes are recruited into the maternal-fetal interface and their role in the processes of term and preterm labor [22].

The current hypothesis is that the chorioamniotic membranes exhibit chemotactic processes driven by specific chemokines in order to recruit maternal circulating

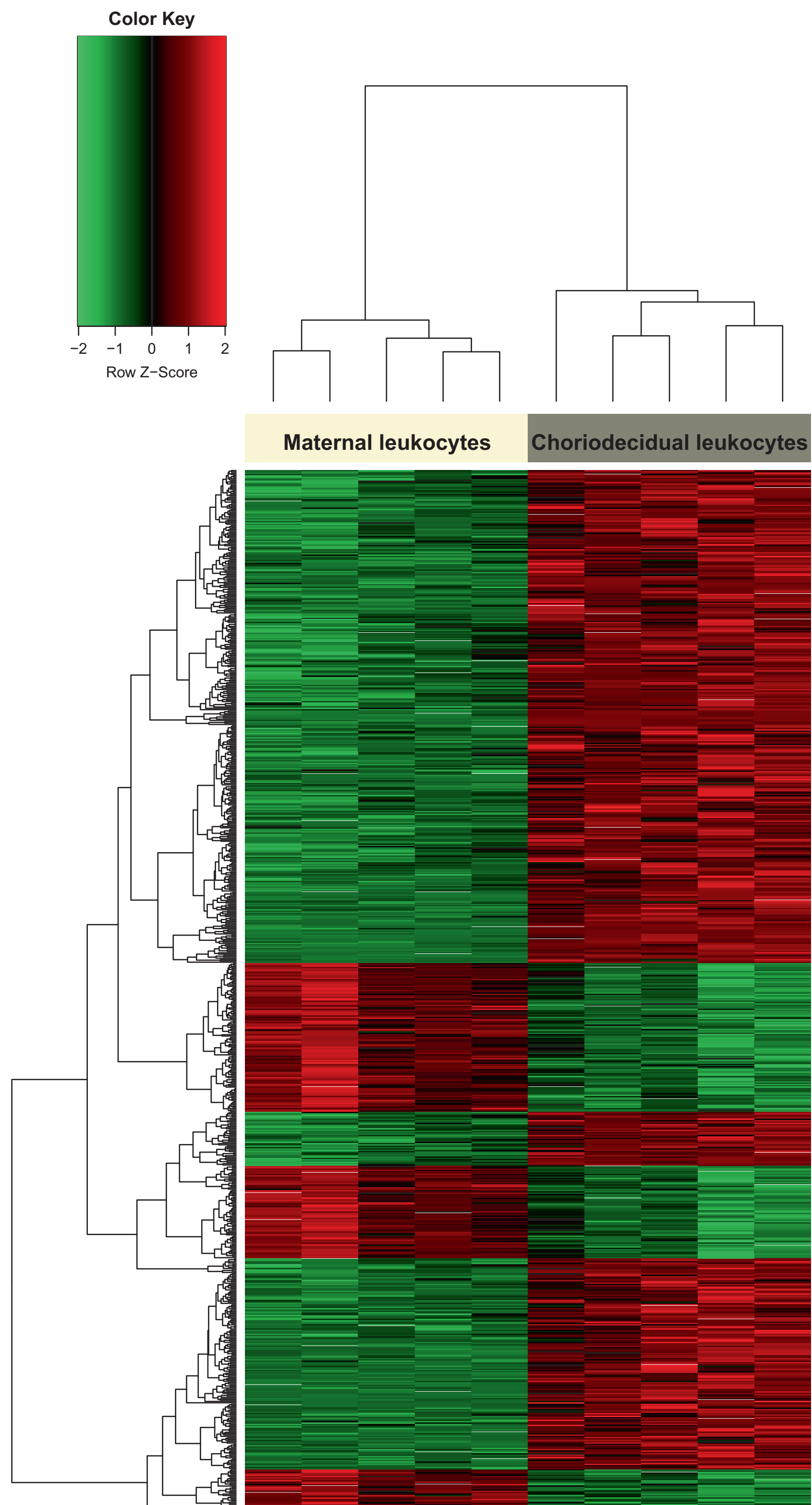

Fig. 1 Heat map representation of the differentially expressed genes in the choriodecidual leukocytes or maternal circulating leukocytes from women who underwent spontaneous labor at term. Heat map displays the unsupervised hierarchical clustering of the 775 genes demonstrating the transcriptomic changes of choriodecidual leukocytes and maternal circulating leukocytes. The negative *z*-score indicates the down-regulated genes (shown in green), and the positive *z*-score indicates the up-regulated genes (shown in red). The labels on the top of the heat map indicate individual women samples ($n = 5$ each)

leukocytes into the choriodecidua [14, 16, 23–25]. In addition, the responsiveness of peripheral leukocytes to be attracted to chemotactic stimuli derived from the chorioamniotic membranes seems to be a key feature in this process [26]. Consistent with such a hypothesis, previous studies have demonstrated that peripheral leukocytes

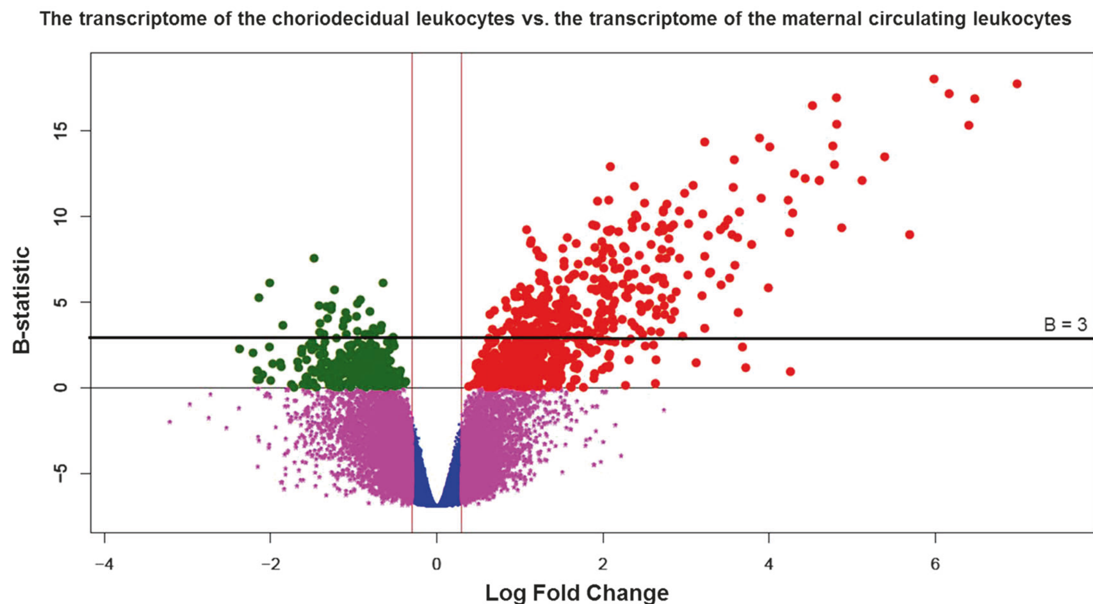

Fig. 2 Volcano plot displaying the differentially expressed genes between choriodecidual leukocytes and maternal circulating leukocytes from women who underwent spontaneous labor at term. B-statistic values are plotted against the log fold change (base 2). A B-statistic of zero corresponds to a 50–50 chance that the gene is differentially expressed (gray line). A B-statistic of ≥3 indicates a 95.3% chance that the gene is differentially expressed (black line). On the *x*-axis, values outside the red lines represent log fold changes of ≥0.3 between choriodecidual leukocytes and maternal circulating leukocytes. Red dots represent the up-regulated genes and green dots represent the down-regulated genes

displayed increased chemotactic activity and cytokine production during the process of labor [27, 28], resembling an inflammatory phenotype similar to that observed in pathological conditions [29, 30]. Collectively, these data suggest that the chorioamniotic membranes, and most likely the decidual stromal cells, recruit maternal circulating leukocytes into the choriodecidua prior to and during the process of labor. Following recruitment, the choriodecidual leukocytes expressed cell adhesion molecules (CAMs) [16, 31–33] and labor mediators including cytokines/chemokines [14–16, 34] and matrix metalloproteinases (MMPs) [35]. All of which elicit cell-mediated immune responses that participate in the process of labor [1, 36–38].

All of the above suggests that the maternal circulating leukocytes are activated prior to and during the process of labor and that their activation status may be similar to that expressed by the choriodecidual leukocytes. However, a direct comparison between the choriodecidual leukocytes and maternal circulating leukocytes during the process of labor at term has not been made. The aims of this study were: (1) to characterize the transcriptome of the choriodecidual leukocytes and maternal circulating leukocytes from the same woman undergoing spontaneous labor at term, and (2) to explore the biological processes enriched in these choriodecidual leukocytes.

Results and discussion

To our knowledge, this is the first study comparing the transcriptomic signatures of the choriodecidual leukocytes and maternal circulating leukocytes during the process of labor at term. Our first finding was that the transcriptome of the choriodecidual leukocytes was different from the transcriptome of the maternal circulating leukocytes. The heat map in Fig. 1 shows a mirrored image between the transcriptomic changes of choriodecidual leukocytes and maternal circulating leukocytes; in other words, up-regulated genes in the choriodecidua were down-regulated in the maternal circulation and vice versa. Similarly, the volcano plot (Fig. 2) shows the differentially expressed genes between choriodecidual and maternal circulating leukocytes.

These results demonstrate that there are drastic differences between the transcriptome of the choriodecidual leukocytes compared to the maternal circulating leukocytes during the process of labor at term. Although there is no doubt that the maternal circulating leukocytes are activated prior to and during the process of labor [26–28], the findings, herein, support the concept that the maternal-fetal interface (decidua basalis and choriodecidua or decidua parietalis) is an enriched microenvironment that includes specific leukocyte subsets during the process of labor [22, 33].

Table 1 List of the top 100 differentially up-regulated genes between choriodecidual leukocytes and maternal circulating leukocytes

Name	Symbol	Log fold change (base 2)	Fold change	*B*-statistic	Adjusted *P*-value
C-C motif chemokine ligand 18	*CCL18*	6.98	126.50	17.77	3.7E−09
Glycoprotein nmb	*GPNMB*	6.47	88.89	16.91	9.4E−09
Selenoprotein P	*SEPP1*	6.40	84.51	15.36	8.3E−08
Fibronectin 1	*FN1*	6.17	71.80	17.17	9.3E−09
Ribonuclease A family member 1, pancreatic	*RNASE1*	5.98	63.22	18.02	3.7E−09
Secreted phosphoprotein 1	*SPP1*	5.69	51.59	8.94	2.9E−05
Complement C1q C chain	*C1QC*	5.39	41.93	13.49	7.2E−07
Phospholipid transfer protein	*PLTP*	5.12	34.70	12.08	2.6E−06
C-C motif chemokine ligand 2	*CCL2*	4.87	29.27	9.37	2.3E−05
Cathepsin L	*CTSL*	4.81	28.10	15.40	8.3E−08
Formyl peptide receptor 3	*FPR3*	4.81	28.05	16.94	9.4E−09
Legumain	*LGMN*	4.78	27.54	13.01	1.1E−06
Complement C1q B chain	*C1QB*	4.76	27.18	14.10	3.5E−07
Mannose receptor C-type 1	*MRC1*	4.60	24.33	12.12	2.6E−06
Peripheral myelin protein 22	*PMP22*	4.52	22.94	16.47	1.8E−08
Metallothionein 1G	*MT1G*	4.30	19.71	12.52	1.9E−06
Solute carrier organic anion transporter family member 2B1	*SLCO2B1*	4.28	19.47	10.18	1.3E−05
Epithelial membrane protein	*EMP1*	4.24	18.94	9.03	2.8E−05
Metallothionein 1×	*MT1X*	4.23	18.75	10.96	7.3E−06
V-set and immunoglobulin domain containing 4	*VSIG4*	4.01	16.07	14.08	3.5E−07
T-cell immunoglobulin and mucin domain containing 4	*TIMD4*	3.99	15.89	5.82	3.2E−04
Regulator of G-protein signaling 1	*RGS1*	3.90	14.95	11.10	6.6E−06
Metallothionein 1E	*MT1E*	3.88	14.75	14.56	2.4E−07
C-×-C motif chemokine ligand 10	*CXCL10*	3.79	13.83	8.37	4.6E−05
Membrane spanning 4-domains A4A	*MS4A4A*	3.64	12.48	10.27	1.2E−05
Oxidized low density lipoprotein receptor 1	*OLR1*	3.63	12.35	4.39	8.2E−04
Folate receptor beta	*FOLR2*	3.62	12.30	8.74	3.4E−05
Interferon alpha inducible protein 27	*IFI27*	3.59	12.00	7.18	1.1E−04
Complement C1q A chain	*C1QA*	3.58	11.95	13.32	8.3E−07
Apolipoprotein C1	*APOC1*	3.57	11.85	11.67	3.6E−06
Macrophage scavenger receptor 1	*MSR1*	3.55	11.73	8.94	2.9E−05
Interleukin 6	*IL6*	3.52	11.49	6.41	2.1E−04
Complement C2	*C2*	3.50	11.34	9.80	1.8E−05
CD80 molecule	*CD80*	3.42	10.70	6.03	2.7E−04
Ectonucleotide pyrophosphatase/ phosphodiesterase 2	*ENPP2*	3.41	10.65	9.23	2.5E−05
Matrix metallopeptidase 19	*MMP19*	3.27	9.64	8.86	3.1E−05
C-C motif chemokine ligand 13	*CCL13*	3.22	9.34	7.70	7.7E−05
Metallothionein 1M	*MT1M*	3.22	9.34	3.50	1.6E−03
Nuclear protein 1, transcriptional regulator	*NUPR1*	3.22	9.33	14.32	3.0E−07

Table 1 (continued)

Name	Symbol	Log fold change (base 2)	Fold change	*B*-statistic	Adjusted *P*-value
ADAM like decysin 1	*ADAMDEC1*	3.20	9.18	10.16	1.3E−05
C-C motif chemokine ligand 4	*CCL4*	3.19	9.14	5.40	4.3E−04
Neuropilin 2	*NRP2*	3.08	8.48	11.79	3.4E−06
SLAM family member 8	*SLAMF8*	3.03	8.17	9.57	2.2E−05
Plasminogen activator, urokinase	*PLAU*	3.02	8.12	6.60	1.8E−04
Chromosome 15 open reading frame 48	*C15orf48*	2.96	7.77	3.00	2.2E−03
Malic enzyme 1	*ME1*	2.92	7.57	10.33	1.2E−05
Activating transcription factor 3	*ATF3*	2.92	7.56	7.58	8.4E−05
DAB2, clathrin adapter protein	*DAB2*	2.88	7.36	5.58	3.6E−04
C-C motif chemokine ligand 8	*CCL8*	2.86	7.26	4.46	8.0E−04
Formin like 2	*FMNL2*	2.85	7.20	9.51	2.2E−05
CD163 molecule	*CD163*	2.82	7.07	5.21	4.7E−04
Ral guanine nucleotide dissociation stimulator like 1	*RGL1*	2.81	7.03	7.98	6.2E−05
Sprouty related EVH1 domain containing 1	*SPRED1*	2.81	7.01	9.34	2.3E−05
Neuropilin 2	*NRP2*	2.79	6.92	8.70	3.5E−05
Lipoma HMGIC fusion partner-like 2	*LHFPL2*	2.77	6.81	10.74	8.3E−06
Metallothionein 1A	*MT1A*	2.76	6.78	7.54	8.5E−05
C-X-C motif chemokine ligand 2	*CXCL2*	2.74	6.69	4.31	8.7E−04
Stearoyl-CoA desaturase	*SCD*	2.74	6.69	6.05	2.7E−04
Solute carrier family 7 member 8	*SLC7A8*	2.74	6.66	4.81	6.3E−04
Phospholipase A2 group VII	*PLA2G7*	2.73	6.61	10.36	1.2E−05
Transcobalamin 2	*TCN2*	2.72	6.61	9.15	2.5E−05
Chloride intracellular channel 2	*CLIC2*	2.72	6.61	10.27	1.2E−05
Lamin A/C	*LMNA*	2.72	6.60	6.10	2.6E−04
C-C motif chemokine ligand 3	*CCL3*	2.72	6.57	6.40	2.1E−04
MER proto-oncogene, tyrosine kinase	*MERTK*	2.71	6.55	9.53	2.2E−05
Olfactomedin like 2B	*OLFML2B*	2.71	6.54	8.05	5.9E−05
G protein-coupled receptor 34	*GPR34*	2.71	6.53	5.27	4.5E−04
RasGEF domain family member 1B	*RASGEF1B*	2.68	6.42	3.26	1.8E−03
GDNF family receptor alpha 2	*GFRA2*	2.68	6.41	7.58	8.4E−05
Serpin family E member 1	*SERPINE1*	2.67	6.38	9.12	2.5E−05
Serpin family F member 1	*SERPINF1*	2.66	6.31	6.47	2.0E−04
Epiregulin	*EREG*	2.65	6.26	4.56	7.6E−04
Interferon gamma	*IFNG*	2.64	6.24	4.20	9.5E−04
Nuclear receptor subfamily 4 group A member 1	*NR4A1*	2.61	6.10	6.34	2.1E−04
Amphiregulin	*AREG*	2.58	5.97	3.28	1.8E−03
Hematopoietic prostaglandin D synthase	*HPGDS*	2.58	5.97	8.38	4.6E−05
Metallothionein 1H	*MT1H*	2.56	5.89	4.94	5.9E−04
	PHLDA1	2.56	5.88	5.73	3.2E−04

Table 1 (continued)

Name	Symbol	Log fold change (base 2)	Fold change	*B*-statistic	Adjusted *P*-value
Pleckstrin homology like domain family A member 1					
Fatty acid binding protein 3	*FABP3*	2.53	5.79	5.72	3.2E−04
Heat shock protein family B	*HSPB1*	2.53	5.78	4.72	6.7E−04
Acid phosphatase 5, tartrate resistant	*ACP5*	2.51	5.71	9.43	2.2E−05
CD69 molecule	*CD69*	2.51	5.70	6.94	1.4E−04
Sialic acid binding Ig like lectin 1	*SIGLEC1*	2.49	5.61	5.75	3.2E−04
Solute carrier family 30 member 1	*SLC30A1*	2.47	5.56	3.16	2.0E−03
X-C motif chemokine ligand 2	*XCL2*	2.46	5.51	4.44	8.0E−04
Metallothionein 1F	*MT1F*	2.45	5.46	5.86	3.1E−04
Chemerin chemokine-like receptor 1	*CMKLR1*	2.44	5.44	4.85	6.1E−04
Metallothionein 1 pseudogene 3	*MT1P3*	2.41	5.31	9.93	1.6E−05
Acid phosphatase 2, lysosoma	*ACP2*	2.39	5.23	10.11	1.4E−05
Leukocyte immunoglobulin like receptor B4	*LILRB4*	2.38	5.20	11.77	3.4E−06
Transferrin receptor	*TFRC*	2.37	5.19	6.66	1.7E−04
Tetraspanin 15	*TSPAN15*	2.35	5.11	9.35	2.3E−05
Peroxisome proliferator activated receptor gamma	*PPARG*	2.35	5.09	9.68	2.0E−05
Solute carrier family 1 member 3	*SLC1A3*	2.35	5.09	3.83	1.2E−03
Colony stimulating factor 1	*CSF1*	2.34	5.07	5.83	3.1E−04
Serpin family G member 1	*SERPING1*	2.31	4.96	6.65	1.8E−04
Dual specificity phosphatase 10	*DUSP10*	2.31	4.94	5.70	3.3E−04
Solute carrier family 41 member 2	*SLC41A2*	2.30	4.92	7.41	9.5E−05
MAM domain containing 2	*MAMDC2*	2.30	4.91	5.24	4.6E−04
Nuclear receptor subfamily 4 group A member 3	*NR4A3*	2.27	4.82	5.36	4.4E−04

We found that choriodecidual leukocytes overexpressed several genes associated with cell migration and regulation of immune response. A total of 270 genes were up-regulated while only 17 genes were down-regulated in the choriodecidual leukocytes compared to the maternal circulating leukocytes. The top 100 up-regulated genes are displayed in Table 1. The most up-regulated genes (fold change >32) in choriodecidual leukocytes, demonstrated by qRT-PCR (Table 2) were the following: (1) C-C motif chemokine ligand 18 (*CCL18*), (2) glycoprotein nmb (*GPNMB*), (3) selenoprotein P (*SEPP1*), (4) fibronectin 1 (*FN1*), (5) ribonuclease A family member 1, pancreatic (*RNASE1*), (6) secreted phosphoprotein 1 (*SPP1*), (7) complement C1q C chain (*C1QC*), and (8) phospholipid transfer protein (*PLTP*).

These results coincide with previous studies demonstrating that choriodecidual leukocytes express CAMs [16, 39, 40], cytokines [8, 41, 42], chemokines [16, 34], and their receptors [16, 34, 43], enzymes [35, 44, 45], antimicrobial peptides [46], markers of activation [17, 39,47–49], and even hormone receptors [50] before and during the process of labor. Together, these findings demonstrate that the choriodecidual leukocytes are a rich source of inflammatory mediators that can participate in the pro-inflammatory milieu that accompanies the process of labor.

The current study also demonstrated that the choriodecidual leukocytes from women who underwent spontaneous labor at term overexpressed genes implicated in the process of chemotaxis, including CCL2, CCL3, CCL4, and CXCL10. This finding is in line with previous studies demonstrating that the chorioamniotic membranes (including the decidua) exhibit leukocyte chemotaxis through the expression of these chemokines during the process of labor [14, 23, 25, 26]. These results suggest that besides being a source of pro-inflammatory mediators, the choriodecidual leukocytes can actively participate in the process of

Table 2 qRT-PCR validation in selected genes differentially expressed in the choriodecidual leukocytes and maternal circulating leukocytes

Gene name	Gene symbol	qRT-PCR ($n = 9$)		
		Higher median expression in	Fold change (median)[a]	*P*-value[b]
C-C motif chemokine ligand 18	*CCL18*	Choriodecidual leukocytes	5691.58	<0.001
Glycoprotein nmb	*GPNMB*	Choriodecidual leukocytes	477.82	<0.001
Selenoprotein P	*SEPP1*	Choriodecidual leukocytes	1507.69	<0.001
Fibronectin 1	*FN1*	Choriodecidual leukocytes	1198.83	<0.001
Ribonuclease A family member 1, pancreatic	*RNASE1*	Choriodecidual leukocytes	952.14	<0.001
Secreted phosphoprotein 1	*SPP1*	Choriodecidual leukocytes	157.86	<0.001
Complement C1q C chain	*C1QC*	Choriodecidual leukocytes	439.91	<0.001
Phospholipid transfer protein	*PLTP*	Choriodecidual leukocytes	466.74	<0.001

Genes with a differential expression of logFCh > 5 and $B > 3$

[a]Fold change calculated with the $2^{-\Delta\Delta Ct}$ method

[b]Statistical differences were analyzed using Mann-Whitney *U* tests

leukocyte recruitment during the process of labor. In other words, this data suggests that leukocytes infiltrating the choriodecidua trigger a positive feedback mechanism whereby more leukocytes are recruited into the maternal-fetal interface.

To gain a deeper understanding of the biological processes up-regulated in the choriodecidual leukocytes from women who underwent spontaneous labor at term, analysis of functional protein association networks was performed with the eight most up-regulated genes (Supplementary Figure 2). Such analysis demonstrated that these genes do not have significant interactions. This means that the interactions between these genes/proteins are unknown or that they do not have a meaningful biological interaction. Since we did not find interactions among the eight most up-regulated genes, gene ontology was performed in all of the up-regulated genes in the choriodecidual leukocytes. Figure 3 shows that the following biological processes were enriched in the choriodecidual leukocytes from women who underwent spontaneous labor at term: (Group 1) cell migration and regulation of immune response, (Group 2) chemotaxis, and (Group 3) humoral immune response.

The most up-regulated of these biological processes was the humoral immune response pathway, including T-cell and B-cell responses. Previously, we demonstrated that the chorioamniotic membranes can recruit T cells, which are implicated in the physiological [14–16] and pathological [51–55] processes of labor. Indeed, in vivo activation of T cells through the CD3 complex induces premature labor and delivery [56]. B cells can also be recruited by the chorioamniotic membranes [14] and constitute a portion of the choriodecidual leukocytes [16, 57, 58]. The role of B cells during pregnancy and its complications have been widely studied by Jensen and collaborators [59–68]. Taken together, these data provide further evidence that the adaptive limb of immunity is involved in the process of labor.

Choriodecidual leukocytes up-regulated the expression of genes involved in the complement activation pathway. Such a pathway has been implicated in susceptibility to viral infections [69] and the pathological process of preterm labor [70–74], but not in the physiological process of labor at term. Therefore, this finding requires further investigation.

The fact that choriodecidual leukocytes express transcripts implicated in the humoral immune response pathway is in line with previous reports indicating that visfatin, a pre-B-cell colony enhancing factor, is expressed in the decidua [75] and amniotic fluid [76] during the physiologic and infection-related pathologic processes of labor. Together, these data suggest that the humoral immune response pathway is implicated in the mechanisms of labor amplification and the host defense response against intra-amniotic infection.

Interestingly, the choriodecidual leukocytes over-expressed the peroxisome proliferator-activated receptor gamma (PPARγ) during the process of labor. PPARγ is expressed in the choriodecidual tissues [77], including decidual macrophages [78], where it was suggested to participate in the process of labor [79]. Recently, we reported that PPARγ activation (via rosiglitazone) attenuates the pro-inflammatory response at the maternal-fetal interface induced by a microbial product [78] or activation of invariant natural killer T cells [55]. Altogether, these data suggest that the choriodecidual leukocytes express PPARγ in order to dampen the pro-inflammatory microenvironment that accompanies the process of labor.

A limitation of the current study is that the isolated choriodecidual leukocytes had a moderate purity (>70%), which suggest that the gene expression reported, herein,

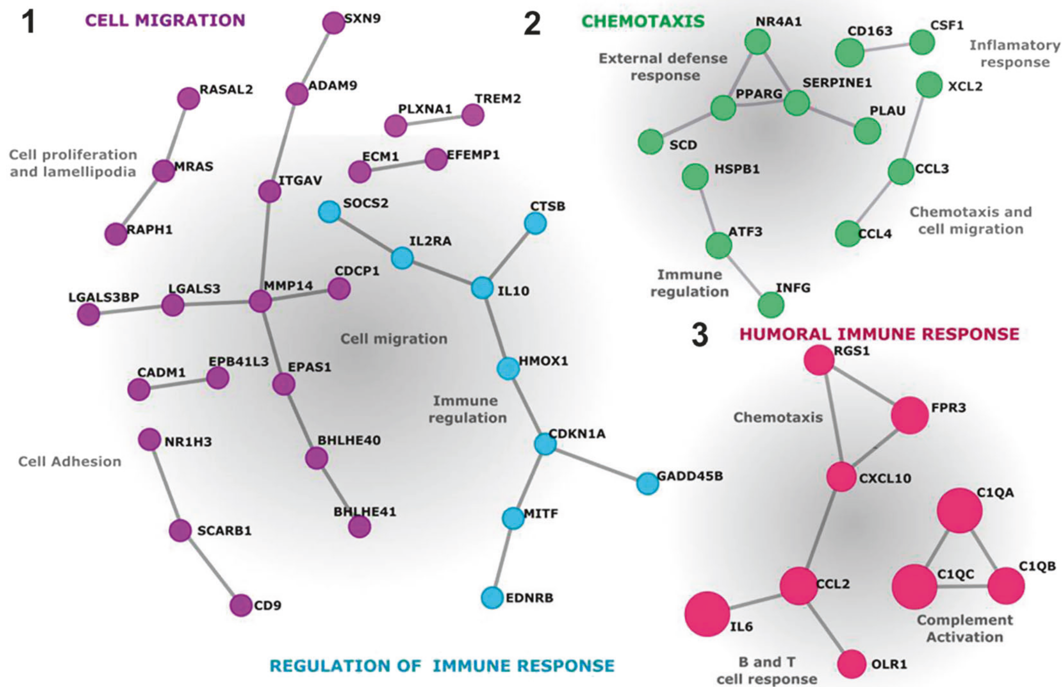

Fig. 3 STRING-based analysis representing up-regulated genes in the choriodecidual leukocytes from women who underwent spontaneous labor at term. The interaction networks show significantly up-regulated transcripts (fold change >2 and adjusted $p < 0.001$) from microarray data. For functional enrichment analysis, the up-regulated genes were divided into three categories based on their fold change: Group 1: fold change >2 and <5, Group 2: fold change >5 and <10, and Group 3: fold change >10

may also include the transcripts expressed by decidual and stromal choriodecidual cells. Yet, most of the mediators reported herein are involved in immune responses.

In conclusion, this study demonstrated that there are transcriptomic differences between the choriodecidual leukocytes and maternal circulating leukocytes during the process of labor at term. The biological processes enriched in the choriodecidual leukocytes during the process of labor are: (1) cell migration and regulation of immune response, (2) chemotaxis, and (3) humoral immune response, which includes T-cell and B-cell responses. Collectively, these data show that there are striking differences between the transcriptome of the choriodecidual leukocytes and the transcriptome of maternal circulating leukocytes. The interaction network analysis revealed that the choriodecidual leukocytes are enriched in immune mediators implicated in the spontaneous process of labor at term.

Materials and methods

Human samples and clinical definitions

This study was approved by the Internal Review Board of the Instituto Nacional de Perinatología in Mexico City (Register number 212250-02191). Maternal peripheral blood samples were collected within 30 min before delivery and the chorioamniotic membranes immediately after delivery from the same woman. Participants included healthy women who underwent spontaneous labor at term (>37 weeks of gestation, calculated from the date of the last menstrual period) resulting in delivery. Labor was defined by the presence of regular uterine contractions and cervical changes (>2 cm). Five participants were included in the first phase of the study (microarrays) and nine participants were included in the second phase of the study (qRT-PCR). The demographic characteristics of the study population are shown in Table 3.

Isolation of maternal leukocytes

Maternal circulating leukocytes were isolated using a density gradient (Polymorphprep; Axis-Shield, Oslo, Norway), according to the manufacturer's instructions. Mononuclear and polymorphonuclear leukocytes were collected and washed with 1× phosphate buffered saline (PBS). Red blood cells were lysed by incubating the leukocyte suspensions with erythrocyte lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) for 15 min at room temperature. Leukocyte suspensions were

Table 3 Demographic characteristics of the study population

	Microarrays ($n = 5$)			qRT-PCR ($n = 9$)	Microarrays vs. qRT-PCR (P-value)[b]
	Maternal circulating leukocytes	Choriodecidual leukocytes	P-value[a]		
Maternal age	32 (20–39)	31 (16–33)	0.310	26 (18–32)	0.315
Gestational age (weeks)	39.4 (37.2–41.3)	39.4 (37.2–41.3)	0.690	39.0 (37.2–41.3)	0.968
Number of pregnancies	2.5 (1–4)	2.5 (1–4)	0.686	2 (1–4)	0.442
Number of live births	2.0 (1–4)	2.0 (1–4)	0.686	1.5 (1–3)	0.645
Newborn's sex	Male = 2 Female = 3	Male = 3 Female = 2	0.527	Male = 5 Female = 4	0.809

Values are presented as medians (range). Differences were assessed using Mann-Whitney *U* test for all variables, except for Newborn's sex where *Chi*-square test was used

[a]Differences between maternal circulating leukocytes and choriodecidual leukocytes samples used in microarrays

[b]Differences between microarray samples and qRT-PCR samples

then washed with 1× PBS and the resulting cell pellet was resuspended in RNA*later* solution (Ambion, Life Technologies, Carlsbad, CA, USA) and stored at −80 °C. The entire process of isolation was performed using ice-cold buffers in order to minimize RNA degradation.

Isolation of choriodecidual leukocytes

Following collection of the chorioamniotic membranes, the amnion was separated from the chorion manually. Next, the entire chorion was cut into fragments (approximately 3 × 3 cm), which were placed into a flask containing 50–100 mL of a digestion solution [Dubelcco's Modified Eagle Medium containing 0.1% of trypsin]. The flask was then incubated at 37 °C for 5–10 min with low agitation. The volume of the digestion solution and the time of incubation depended on the size of the chorion. After incubation, the tissue suspension was filtered using sterile gauze and the flow-through was collected. The resulting cell suspension was washed with 1× PBS and red blood cells were lysed using the erythrocyte lysis buffer, as previously described. Choriodecidual leukocytes were then isolated from other choriodecidual cells present in the cell suspension by magnetic cell sorting. Briefly, cell suspensions were incubated with a CD45 microbead-conjugated antibody and leukocytes (CD45+ cells) were isolated, according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). Purified choriodecidual leukocytes were stored at −80 °C in RNA*later* solution. The purity of choriodecidual leukocytes was >70% of CD45+ cells (Supplementary Figure 1). The entire process of isolation was performed using ice-cold buffers to minimize RNA degradation.

RNA isolation

The TRIzol® reagent (Invitrogen, Life Technologies Corporation, Grand Island, NY, USA) was used to extract the total RNA from RNA*later* solution-stored leukocytes, following the manufacturer's instructions. RNA purity and concentration were assessed with the 6000 NanoDrop® spectrophotometer, and RNA integrity was evaluated with the Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA). RNA integrity numbers (RIN) were >7.

cDNA synthesis

For microarray experiments, 200 ng of total RNA was amplified with the Ambion WT Expression kit, following the manufacturer's instructions (Ambion, Life Technologies, Carlsbad, CA, USA). Newly synthesized complementary (c)DNA was fragmented and labeled with the GeneChip® WT Terminal Labeling kit (Affymetrix, Santa

Clara, CA, USA), according to the manufacturer's instructions.

For qRT-PCR experiments, 500 ng of total RNA was used to generate cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Mannheim, Germany) and random hexamers as primers. cDNA synthesis was carried out in a Mastercycler Gradient Thermalcycler (Eppendorf, Hamburg, Germany). Next, cDNA was stored at −20 °C until use.

Microarray analysis

Five sets of matched samples from choriodecidual leukocytes and maternal circulating leukocytes were utilized for microarray analysis (Table 3). The GeneChip® Human Gene 1.0 ST array (Affymetrix Inc., Santa Clara, CA, USA) was used to measure the gene expression levels in each specimen, following manufacturer's instructions. This array included 28,869 well-annotated genes with 764,885 probes.

Real-time quantitative polymerase chain reaction

To validate the gene expression results from the microarray, eight up-regulated genes in choriodecidual leukocytes compared to maternal circulating leukocytes and two reference genes (Supplementary Table 1) were selected for qRT-PCR analysis. Validation was performed using a set of nine matched samples (choriodecidual leukocytes and maternal circulating leukocytes from nine different women). The expression analysis was performed by using the 48.48 Dynamic Array integrated fluidics chips (BioMark Fluidigm Corporation, San Francisco, CA, USA), following the manufacturer's instructions.

Statistical analyses

Raw microarray data were background-corrected using the Robust multi-array average (RMA) method [80], and normalized using the Quantile Normalization method [81]. Differential expression was determined using statistical linear models with arbitrary coefficients; contrasts of interest were analyzed using the bioconductor library limma [82, 83]. Correction for multiple hypotheses was applied using a false discovery rate (FDR). Genes were selected based on a log fold change ≥0.3 and a B-statistic ≥3.

Differential expression between experimental qRT-PCR data was determined with the $2^{-\Delta\Delta Ct}$ method [84]. The qRT-PCR data were non-normally distributed according to the Shapiro-Wilk test. The Mann-Whitney U test was used for comparisons between groups. A P-value ≤ 0.05 was considered statistically significant. The statistical analysis was performed using the SPSS version 20 software (IBM Corporation, North Castle, NY, USA).

Interaction network analysis

The functional network analysis for up-regulated genes in the tested conditions (fold change >2, adjusted $P < 0.001$) was constructed using protein–protein associations using the STRING (Search Tool for the Retrieval of Interacting Genes) v.10.0 software [85]. The network was built using high-quality associations (interaction score ≥0.700). Genes were grouped based on their fold change expression into three different groups: Group 1: fold change >2 and <5, Group 2: fold change >5 and <10, and Group 3: fold change >10. These groups were analyzed separately using the STRING v.10.0 software. Gene ontology annotation was added to the graph based on the functional enrichment analysis results for the analyzed genes.

Data availability

All of the data are available in the tables and figures of the manuscript. Microarray data is available upon request at r.vega@inper.mx / vegarodrig@gmail.com

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Author contributions MAH, NGL, and RVS participated in the experimental design of the study; MAH and JAVP performed the experimental work; VGF, CRE, LMAS, and NMA performed the statistical analyses, and MAH, NGL, VGF, and RVS analyzed and interpreted the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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