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Condensation: Novel integrative view of the placental gene expression, circulating immune profiles, and analysis of the maternal-fetal interface provides evidence of pro-inflammatory shifts in preterm births.

Short Title: Compartment specific pro-inflammatory changes in preterm birth

AJOG at a Glance:

A. Our primary goal was to obtain an integrated view of the maternal and placental contribution to preterm birth to understand the compartment-specific contribution to the etiology of the syndrome.

B. Placental transcriptomic analyses revealed differentially expressed genes that were highly enriched for inflammatory biological processes which were supported through analyses of maternal immune cells, inflammatory mediators in maternal circulation and at the maternal-fetal interface, as well as through histological lesions in the maternal-fetal interface.

C. Distinct profiles in preterm vs term births shed light on changes within the immune system in multiple levels and compartments to help support previously used therapeutics and offer potential new targets to consider in clinical settings.
ABSTRACT

Background: Preterm birth (PTB) remains a leading obstetric complication due to an incomplete understanding of the multifaceted etiology. It is known that immune alterations towards a pro-inflammatory profile are observed in women with PTB, but therapeutic interventions are still lacking due to a scarcity of evidence in the integration of maternal and placental inter-related compartments.

Objective: Our goal was to obtain an integrated view of the maternal and placental contribution to PTB compared to normal term pregnancies for an in-depth understanding of the immune/inflammatory involvement, intending to identify novel strategies to mitigate the negative impact of inflammation.

Study design: We prospectively recruited 79 women with preterm or term deliveries and collected placentas for RNA sequencing (RNAseq), histological analyses and to assess levels of inflammatory mediators. Blood samples were also collected to determine the circulating immune profiles by flow cytometry and to evaluate the circulating levels of inflammatory mediators.

Results: Placental transcriptomic analyses revealed 102 differentially expressed genes (DEGs) upregulated in PTB, including known and novel targets, which were highly enriched for inflammatory biological processes according to gene ontology analyses. Analysis of maternal immune cells revealed distinct profiles in PTB vs term births including an increased percentage of CD3- cells and monocyte subsets and decreased CD3+ cells along with Th17 subsets of CD4+ lymphocytes. Supporting our bioinformatic findings, we found increases in pro-inflammatory mediators in the plasma, placenta, and fetal membranes (primarily the amnion) of women with PTB, such as IL-6 and TNF-α. These findings were not distinct between spontaneous (sPTB) vs iatrogenic PTBs (iPTB) except at a molecular level where sPTB presented with an elevated
inflammatory profile as compared to iPTB. Analysis of placental histology revealed increased structural and inflammatory lesions in PTB vs term births. We found that genes upregulated in placentas with inflammatory lesions have enrichment of pro-inflammatory pathways.

**Conclusions:** This work sheds light on changes within the immune system in PTB on multiple levels and compartments to help identify pregnancies at high risk of PTB and to discover novel therapeutic targets for PTB.

**Keywords:** Preterm birth, Inflammation, Maternal-fetal interface, placenta, maternal circulation, RNA sequencing, transcriptomic profiles
INTRODUCTION

Preterm birth (PTB) is a leading obstetric complication, responsible for nearly 16 percent of all neonatal deaths worldwide, and is a major contributor to neonatal morbidities.\(^1\) PTB has life-long health consequences in surviving newborns, such as neurodevelopmental disorders and cardiovascular diseases.\(^2,3\) Etiology is unknown for a majority of PTB, and mechanisms remain poorly understood, resulting in the lack of efficient preventive and therapeutic options.

Inflammation is essential throughout pregnancy - from early fetal development to labor onset.\(^4,5\) Inflammatory mechanisms are tightly regulated by local/systemic immune cells and mediators in uncomplicated pregnancies. However, evidence shows impaired inflammatory responses in gestational tissues and maternal circulation in pregnancy complications – notably PTB.\(^6-10\)

Inflammation can be induced by infections that are detected in 20-30% of PTB cases.\(^11,12\) PTB can also occur without detectable microorganisms (i.e. sterile inflammation) - whereby endogenous danger signals derived from cellular stress or necrosis, known as damage-associated molecular patterns (DAMPs) or alarmins, are often detected.\(^13-16\) In both instances, local immune responses are triggered, negatively affecting pregnancy and fetal life.\(^7\)

Advances in “omics” have allowed for the discovery of genes and pathways that have increased insights into pregnancy complications.\(^9,17,18\) Bulk transcriptomic analyses have shown large transcript changes in the myometrium and placenta of term versus preterm pregnancies.\(^19,20\) Recent work has identified transcriptome changes in maternal peripheral blood which could provide non-invasive ways for identifying women at risk of PTBs.\(^21\) These studies share the involvement of
immune pathways in PTB however, to understand the immune/inflammatory mechanisms involved, it is crucial to investigate the response of different maternal and fetal components to allow in-depth integration of PTB pathology.

Our study aimed to obtain an integrated view of the maternal and placental contribution to PTB syndrome compared to normal pregnancies to allow an in-depth understanding of the immune/inflammatory contributions and identify women that could benefit from targeted anti-inflammatory therapies. We studied the placental transcriptome, identified maternal circulating immune cell and inflammatory profiles, investigated inflammatory mediators, and performed in-depth histological analyses at the maternal-fetal interface. Through the integration of multiple compartments, we obtained a better understanding of the pathways underlying PTB, and importantly their specific localization, that could be leveraged for new therapeutic interventions as well as biomarkers for future studies.
MATERIALS AND METHODS

Ethics approval and patient data collection

Approval was obtained from the CHU Sainte-Justine (CHUSJ) Ethics Board (No: 2015-840). Women were recruited (n=79) from the CHU Sainte Justine Hospital having either an uncomplicated term birth (n=41: 16 laboring) or PTB (n=38: 22 laboring) and gave written informed consent at the time of their admission for delivery. Birth prior to 37 weeks of completed gestation was considered preterm. Preeclampsia, active infection (defined as clinical symptoms of infection and/or antibiotic treatment within the last 7 days) and congenital malformations were excluded. Demographic and obstetrical data were obtained through the evaluation of patients' clinical files.

Tissue and blood collection

Placental (1 sample per quadrant, defined from umbilical cord), taken in the thickest portion of the placental villi, and fetal membrane biopsies were collected shortly after delivery. Placental samples were kept in RNA later for 24h before being stored at -80°C or directly frozen and stored at -80°C. Placenta, fetal membrane, and umbilical cord biopsies were fixed in 4% paraformaldehyde (PFA) for 7 days, transferred to PBS, and embedded into paraffin for hematoxylin & eosin (HE) staining. Blood samples were obtained from each patient within 24h before delivery, notably between 6-12h of delivery with some collected between 3h (particularly patients that had cesarean deliveries) or between 12-24h. If a patient did not deliver within 24h of blood collection, an additional sample was collected, and the latter used for analysis. Fresh blood was immunolabelled with antibodies for immune cell subpopulations of interest (see below for
details). The remaining blood was centrifuged at 2000 rpm (10 min, 4°C) and plasma was collected, aliquoted, and stored at -80°C.

**Immune cell analysis by flow cytometry**

Whole blood (100μl) was immunolabeled for 15 min at room temperature using the following antibodies: FITC-CD3, APC-Cy7-CD4, PerCP-Cy5.5-CD8, PE-CD127, V500-CD19, PE-CF594-CD183, PE-Cy7-CD196, BV605-CD25, BV421-CD194, Alexa Fluor 700-CD14, and APC-CD56 (all from BD Biosciences, ON, Canada). Red blood cells were lysed (0.06 M NH₄Cl, 4 mM KHCO₃, 0.052 mM EDTA), PBMCs washed with PBS, PFA-fixed, and analyzed using a flow cytometer (Fortessa BD Biosciences, ON, Canada). A minimum of 300,000 events were acquired per sample. Data analysis was performed using FlowJo (Tree Star, OR, USA) (see gating strategy Figure S3).

**Inflammatory mediator detection in the maternal plasma and at the maternal-fetal interface**

A panel of cytokines/mediators were analyzed (i.e. Interleukin (IL)-1beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), S100A8, C-Reactive Protein (CRP) and IL-1 receptor antagonist (IL-1Ra)), in placental tissues and plasma via ELISA. Additionally, ELISA analyses were performed for interferon-gamma (IFNγ) and IL-4 in the placenta only whereas IL-8 and progesterone were analyzed in plasma only (all from R&D Systems, Canada except for progesterone: Cayman chemical, USA), (R&D Systems, Canada), as previously described ²².

**RNA sequencing (RNA-seq) and bioinformatic analysis**
Bulk RNA-seq of placental tissues was run in 3 batches (adjusted for). mRNA was extracted using the RNeasy mini kit (Qiagen, Canada) following manufacturer instructions, quantified (Nanodrop 8000, Thermo Fisher Scientific, Canada) and the quality was assessed using the RNA Integrity Number (RIN ≥ 7; Agilent 2100 Bioanalyzer, USA). Libraries were created using the TruSeq RNA Library Kit v2 (Illumina, USA) and fragment sizes were validated on a Bioanalyzer (Agilent 2100, USA). Libraries were sequenced using the HiSeq 4000 system (Illumina, USA) at the CHUSJ. RNA-seq data has been analyzed in accordance with the Genome Analysis Toolkit (GATK) best practices. Alignment to the hg19 (GRCh37) genome reference was performed using STAR aligner, gene expression was measured with the HTseq software using the Ensembl version 75 gene coordinates. Bioinformatic analyses were performed with RStudio version 1.1.463. Data were adjusted for batch effects and sex. Differential gene expression (DEG, adj.p-value<0.05, log fold change (logFC)±1) analyses were achieved with the limma R package. Gene ontology for over-expression analysis is powered by PANTHER through the ClueGO plug-in within Cytoscape. Gene set enrichment analysis (GSEA) and pathway analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed in RStudio using clusterProfiler.

Histological analysis

Five micrometer thick sections from the placenta (at least 3 samples) and fetal membranes were processed for HE staining and evaluated by a histopathologist blinded to pregnancy outcomes assessed according to the Amsterdam criteria, as previously described. Images were captured using a slide scanner (Axioscan, Zeiss, ON, Canada).

Statistical analysis
Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA with Dunnett’s multiple comparisons, Sidak’s multiple comparisons, or Fisher’s exact test, as appropriate, as well as unpaired t-tests and simple linear regressions using GraphPad Prism Version 9.1.2, (GraphPad Software, CA, USA). A probability value of <0.05 was considered statistically significant.
RESULTS

Population characteristics: demographic and obstetrical information

Seventy-nine women were included in this study (41 term, 38 preterm). Demographic and obstetrical characteristics are shown in Table 1. Maternal age differed between groups where term births were older (31.0 ± 1.0 years vs 33.7 ± 0.67 years, p<0.05). Both groups included predominantly Caucasian women with similar pre-pregnancy BMI and family or personal history of hypertension and diabetes. PTBs were more likely to be primiparous (65.5 ± 2.0% vs 35.5 ± 9.5%, p<0.05), had lower gestational age at delivery (32.4 ± 0.5 years vs 39.8 ± 0.1 years p<0.01) and lower birth weight (1666.5 ± 88.0g vs 3534.0 ± 53.0g in term births, p<0.01) with 42% of small for gestational age (SGA) compared to none in term births. Both groups had an equal proportion of labor inductions, cesarean delivery as well as male/female newborns.

Bulk RNA-sequencing of the placenta revealed novel genes involved in inflammatory pathways associated with PTB

To deepen our understanding of the transcriptional profiles, we performed RNAseq on the placentas of women with term and preterm births. Unbiased analyses included 39,193 genes, from which we extracted 331 DEGs (adj. p<0.05, logFC±1) between term and PTB conditions. Of these DEGs, 102 were upregulated and 229 were downregulated in PTB (Figure 1). Within the upregulated genes, many are highly involved in immune regulation (Table 2). An example of a downregulated gene is gamma-aminobutyric acid type A receptor subunit beta1 (GABRB1, logFC= -2.31, adj.p-value=11.28x10^-9), also seen to be negatively modulated in PTB by Paquette et al, supporting our findings\textsuperscript{20}. 
To explore biological specializations and decipher enrichments, we used Gene Ontology (GO, FDR p<0.05) and found up-regulated genes had processes related to inflammatory pathways whereas downregulated genes had no statistically enriched biological processes (Figure 2A). We further removed redundancy using Cytoscape and found that the main pathways affected in PTB are processes related to lymphocyte chemotaxis, neutrophil migration, and antibacterial humoral response (Figure S1). To strengthen our findings, we looked at gene set enrichment (GSEA) and pathway enrichment (KEGG), and both supported the enrichment of immune and inflammatory processes in PTB (Figure 2B-D). We identified dysregulated genes within these enriched pathways (ex. upregulated 4-1BBL, in the cytokine-cytokine receptor interactions identified through KEGG analysis (Figure S2)). Following this work, which highlighted immune-related pathways in PTB, we further investigated which components of the immune system might be at play.

The maternal circulating immune cell profile is altered in preterm as compared to term deliveries

Of the circulating immune cell subpopulations studied, significant changes were observed for both CD3+ and CD3- cells in our patient groups (Figure 3). CD3- cells were increased in PTB (52.9 ± 13.0% vs 40.9 ± 14.0%, p<0.001, Figure 3A) of which the monocyte subtype was increased (46.2 ± 12.9% vs 34.3 ± 18.9%, p<0.01, Figure 3B, E). Other CD3- subsets such as B-lymphocytes and NK cells remained unchanged (data not shown). On the other hand, CD3+ cells were decreased in PTB (46.8 ±13.0% vs 58.7 ±14.0%, p<0.001, Figure 3C) with decreased Th17 subset (4.1 ± 2.1% vs 5.6 ± 2.8%, p<0.05, Figure 3D). Other CD3+ subsets such as CD8+, CD4+, Treg, NK-T, and T-helper cells remained unchanged (data not shown).
Pro-inflammatory mediators are elevated within the maternal circulation and at the maternal-fetal interface

A detailed analysis of the immune mediators in the maternal circulation revealed important differences in PTB. None of the classical cytokines studied showed significant changes (namely IL-1β, IL-6, TNF-α, IL-10, MCP-1, S100A8, IL-8, and IL-1Ra, data not shown). However, when looking at other inflammation-related mediators, we found that progesterone was decreased (89 ± 10ng/mL vs 297 ± 32ng/mL, p<0.0001, Figure 4A; none of the patients were treated therapeutically with progesterone) whereas c-reactive protein (CRP) was increased (7.2 ± 1.2 ug/mL vs 2.4 ± 5.0ug/mL p<0.001, Figure 4B) in the maternal circulation in PTB.

Analysis of the immune mediators at the maternal-fetal interface revealed increased levels of pro-inflammatory IL-6 (1.50 ± 0.20pg/mg vs 0.90 ± 0.08pg/mg, p<0.01, Figure 4C) and TNF-α (2.50 ± 0.38pg/mg vs 1.60 ± 0.20pg/mg, p<0.05, Figure 4D) in PTB, which was correlated to gestational age for IL-6 only (Figure S4). Within fetal membranes, we found that both amnion/chorion showed elevated levels of MCP-1 in PTB (155.2 ± 34.0 pg/mg vs 43.5 ± 8.9 pg/mg; 198.5 ± 35.6 pg/mg vs 71.0 ± 9.7 pg/mg, p<0.05, respectively, Figure 4E). In addition, specific mediators were found altered only in the fetal facing amnion such as increased CRP (59.0 ± 10.7ng/mg vs 24.0 ± 4.4ng/mg p<0.05, Figure 4F) and S100A8 (1.50 ± 0.33ng/mg vs 0.20 ± 0.04ng/mg, p<0.05, Figure 4G), alongside a decrease in IL-6 (124.1 ± 41.7pg/mg vs 427.7 ± 113.4pg/mg, p<0.01, Figure 4H) in PTB.

Histological analysis revealed an increase in placental lesions in preterm births

Placentas from PTBs had elevated structural lesions compared to term deliveries (60.5 vs 14.6 %, p<0.0001) - these included, maternal/fetal vascular malperfusion, accelerated villous maturation,
increased syncytial knots, and fibrin (Figure 5A-B, Table S1). There were additionally more inflammatory lesions in PTB placentas (31.6 vs 12.2%, p=0.05), such as deciduitis (Figure 5B). On the other hand, term placentas were largely found to be lesion free (7.9 vs 73.0%, p<0.0001) (Figure 5B).

Integrated analysis of the PTB syndrome

Considering that PTB is a syndrome with varied etiologies, we first addressed if the differences we observed were detected between spontaneous and iatrogenic deliveries. Within our preterm subset (n=38) we had 18 spontaneous (sPTB) and 20 iatrogenic deliveries (iPTB; 7 were induced but only 3 delivered vaginally). No difference was observed in the maternal immune cells, nor plasma/placenta inflammatory mediators between the subgroups of PTB (data not shown). However, chorionic membranes show an elevation in CRP and TNF-α in sPTB (Figure 6A, B). Additionally, from our transcriptomic data (above), we observed that clustering was equally distributed (Figure 6C), but there were differences between the genes from sPTB vs iPTB placentas. 310 DEGs were found between sPTB vs iPTB, of which 125 were up-regulated and 185 were down-regulated in sPTB (Figure 6D). Importantly, only the upregulated DEGs in sPTB showed over-representation for inflammatory pathways (Figure 6E).

Alongside, we investigated if differences in gene expression were associated with the presence of lesions within the placenta. We looked at gene expression changes between patients in our entire cohort (Figure 7A), specifically within the PTB or term (Figure 7B-C). We found that placentas with inflammatory lesions had significant DEGs, and these showed an over-representation in immune pathways (Figure 7D). On the other hand, structural lesions showed no DEGs compared to placentas without lesions.
Principal findings: We investigated the transcriptional changes in the placenta concerning preterm vs term births alongside the immune/inflammatory profiling within the maternal circulation and the maternal-fetal interface. We found 102 upregulated DEGs in PTB, which were enriched for inflammatory pathways through GO, GSEA, and KEGG analyses. In the maternal circulation, we observed increased CD3- cells and monocytes and decreased CD3+ and Th17 cells, both involved in the pro-inflammatory response. Furthermore, CRP was elevated whilst progesterone was decreased. Similarly, a pro-inflammatory bias was observed at the maternal-fetal interface, with elevated levels of IL-6 and TNF-α in the placenta along with MCP-1, CRP and S100A8 increased predominantly in the fetal facing amnion. Interestingly, strong differences were seen in the transcriptional profiles and proteins between sPTB and iPTB, suggesting that changes are associated with the etiology of PTB.

Results in the context of what is known: Within the 102 upregulated DEGs in PTB, inflammatory pathways were the most significantly enriched using GO, GSEA, and KEGG, which is in line with what others reported. Some upregulated genes we observed, such as bridging integrator 2 (BIN2), have previously been shown to be elevated in PTB, strengthening its potential involvement. Many other genes have important roles in inflammatory processes, such as ADAM9 and CCL20, which are involved in the recruitment of Th17 and Tregs to sites of inflammation. In our study, eleven genes related to neutrophils, which help establish proper local inflammation, were upregulated in the placenta from PTBs. These included dipeptidase 1 (DPEP1), serum amyloid A1 and 2 (SAA1/2), platelet factor 4 (PF4), pro-platelet basic protein (PPBP), proteinase
3 (PRTN3), and C-C chemokines. The latter of which is also found upregulated in the enriched proinflammatory pathways (Table 2) but the actual number and function of neutrophils in PTB remains to be further elucidated. Potential markers were also identified at the protein level, such as changes in IL-6 and TNF-α, alongside increased CRP, all suggested to be associated with increased risks of neonatal complications. In addition, we observed decreased progesterone, which normally increases during pregnancy and is strongly anti-inflammatory, suggesting possible involvement in premature labor.

In terms of immune cell profiles in the maternal circulation, we found decreased CD3+ cells and their Th17 subset, which contrasts with growing evidence that some pregnancy complications are linked to increasing Th17 cells in the periphery. On the other hand, CD3- cells along with their monocyte subset were elevated in PTB, which is in line with previous studies. Monocytes are a major source of cytokines in the inflammatory phase of early pregnancy, however, they are known to change throughout pregnancy - consistent with “activation”. Even though circulating monocytes were increased in PTB, future work is needed to decipher their activation status in the placenta.

Clinical implications: Our study aims to find new therapeutic targets for clinical use and can also be used to support currently used therapeutics, for example, our observed decrease in progesterone supports its use in high-risk women. It will be interesting to further study the components of identified dysregulated signaling pathways to identify novel therapeutic targets (ex. 4-1BBL is enriched in PTB and has been studied as an anti-cancer target (see supplementary figure 3)). Other genes such as DPEP1 and TMIGD3, involved in neutrophil recruitment and NFκB inhibition
respectively, are to our knowledge newly associated with PTB and will be interesting in future work. Although a diagnosis of high-risk pregnancy has to occur before delivery, an in-depth analysis of the placenta could help to identify subgroups with/without inflammation to adapt therapeutic strategies to mitigate the impact of inflammation postnatally on the baby’s development.

**Research implications:** Our study has generated a large transcriptional repertoire pertaining to the changes occurring at the maternal-fetal interface in both term and preterm labor. We have identified genes and pathways dysregulated in PTB, and these specific signatures could be used to identify women who would benefit from anti-inflammatory therapeutic interventions alongside continuing to integrate findings from multiple interrelated compartments to elucidate their involvement in preterm labor.

**Strengths and Limitations:** Our study has equivalent population groups, and we are combining many techniques, and several compartments, which strengthens our findings. By design, term pregnancies were used as controls since there is no ethical way to get gestational age match tissues from healthy pregnancies. However, we previously reported that the inflammatory changes associated with term physiological labor were minimal when compared to pathological pregnancies such as preterm birth. Since the placenta grows throughout gestation, it might cause changes in gene expression levels however, we do not find correlations to gestational age (unless mentioned), which is supported by other studies, showing that PTB cannot be divided into gestational age clusters. Differences in gene expression related to fetal sex, however, are well known and we adjusted for it, as has previously been done. To our surprise we did not see
alterations in classical cytokines, other than IL-6 and TNF-α, in PTB versus term pregnancies, which could be because we excluded women with active infections and therefore might be detecting primarily sterile inflammatory processes rather than pathogen-associated inflammation. Another limitation to our study is the fact that some patients were in labor at the time of blood collection, both in the PTB and term groups, however no differences in circulating cytokines were observed in the subgroups of labor vs no-labor (data not shown). Furthermore, our PTB group had an elevated proportion of SGA neonates, most of which had iatrogenic preterm delivery, which could be contributing to the differences observed.

**Conclusions:** Our work sheds light on changes in placental transcriptomes, immune cell populations, immune mediators in maternal circulation and at the maternal-fetal interface as well as histological lesions in the placenta in PTB and indicates a striking contrast that points to aberrant inflammation in PTB. We additionally identified novel pathways enriched in spontaneous versus iatrogenic preterm births and identified transcriptomic changes concerning placental lesions. A better understanding of these changes will be beneficial to identifying pregnancies at high risk of PTB to develop novel therapeutic targets and subsequently promote neonatal health.

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**AUTHOR’S ROLES**
Camille Couture: Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. Marie-Eve Brien: Data curation, Writing - review & editing. Ines Boufai: Data curation, Formal analysis. Cyntia Duval: Data curation. Dorothee Dal Soglio: Data curation. Elisabeth Ann L. Enninga: Project administration, Writing - review & editing.

Brian Cox: Conceptualization, Project administration, Writing - review & editing. Sylvie Girard: Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing - original draft.

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537 Figure legends
**Figure. 1** Differentially expressed genes (DEGs) between placentas from term vs preterm birth. We identified 331 DEGs between term and PTB, of which 229 were downregulated (blue) and 102 upregulated (red) (A). The top 50 most differentially expressed genes (sorted by logFC) are shown as a row scaled heatmap, where up-regulated genes are in red and down-regulated genes, are in blue (B). Examples of highly up-regulated (C) and down-regulated (D) genes extracted from the heatmap identify potential future targets. ***=p<0.001 by moderated t-statistics in R. A cut-off criteria of p<0.05 and logFC±1 was used for DEG analysis.

**Figure. 2** Altered pathways in PTB. Biological processes using up-regulated DEGs obtained from Gene Ontology (GO) analysis (p<0.05, logFC±1) showed an over-representation of immune-related and inflammatory pathways in preterm birth (FDR <0.05) (A). Gene set enrichment analysis (GSEA, p<0.05, min gene set=3, max gene set=800, 10000 permutations) of all DEGs support GO analysis with activation of immune and inflammatory pathways, and suppression of housekeeping processes (B). Pathway enrichment analysis (KEGG, p<0.05, min gene set=3, max gene set=800, 10000 permutations) additionally found enrichment for immune pathways (C) where you can see an example of the enrichment of genes within the cytokine-cytokine receptor interaction pathways (D).

**Figure. 3** Immune cells were found in maternal blood shortly antepartum using FACS. An increase in CD3- cells in PTB can be seen as a percentage of live cells in the bar graph and through the shifts of CD3 expression in representative participants in the histogram (A). Monocytes were also increased in PTB as a percentage of CD3- cells and through the shift in CD14 expression seen in the histogram (B) and the bivariate contour overlay (E). A decrease in CD3+ cells in PTB can
be seen as a percentage of live cells in the bar graph and through the shifts of CD3 expression in representative participants seen in the histogram (C), Th17 cells were also decreased in PTB as a percentage of CD4+ lymphocytes and through the shift in CD196 expression (D) and the bivariate contour overlay (J). Results are presented as mean ± SEM. * = P < 0.05, ** = P < 0.01, ***=p<0.001 by Unpaired t-tests used for statistical analysis.

Figure. 4 Levels of immune mediators in the maternal circulation and at the maternal-fetal interface. Women with preterm birth (PTB) showed a decrease in progesterone (A) and an increase in CRP (B) in maternal circulation before delivery. Within the placenta, PTB had significantly increased levels of IL-6 (C) and TNF-α (D). Fetal membranes were analyzed separately (amnion and chorion) and there was an increase in MCP-1 in both compartments (E). Only the amnion showed increases in CRP (F) and S100A8 (G) and a decrease in IL-6 (H). Data presented as mean ± SEM. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.001. Unpaired t-tests were used for statistical analysis.

Figure 5: Histological analysis of placentas/fetal membranes from term and preterm deliveries. Representative images of normal and lesioned placenta/fetal membranes (A) including normal villi (1) and normal fetal membrane (2) from term placenta. The most frequent inflammatory lesions included maternal inflammatory response (3) and deciduitis (4) and the most frequent structural defects included excess syncytial knots (5) and excess fibrin/avascular villi (6). Scale bar: 100um. The percentage of structural and/or inflammatory lesions in term and preterm patients demonstrate that preterm deliveries had more structural and inflammatory lesions and fewer lesion-free placentas compared to term deliveries (B).
Figure 6. Gene expression based on spontaneous versus iatrogenic preterm birth. Boxplots show an increase in proinflammatory TNF-α (7.3 ± 1.7 vs 2.6 ± 0.8, p<0.05, A) and CRP (79.4 ± 19.28 vs 25.2 ± 9.6, B) in sPTB versus iPTB. Multidimensional scaling plot shows clustering within PTB patients, sPTB (n= 18, red) versus iPTB (n= 20, blue; out of which 7 were induced but only 3 had vaginal deliveries) (C). Row scaled heatmap of the top 50 DEGs (p<0.05) in order of logFC between sPTB and iPTB show the pattern of gene expression (D). Gene ontology pathways from all the up-regulated DEGs (red) and the down-regulated DEGs (blue) between sPTB and iPTB indicate that immune and inflammatory pathways are over-represented in sPTB (E).

Figure 7. Gene expression based on placental lesions. Placenta were classified as having either inflammatory (In, red), structural (Str, green,) or none (No, blue) lesions. Multidimensional scaling plots show that placental lesions are not clustering individually: Plots showing the clustering of placental lesions within all patients (n=79) (A) as well as those within preterm births (n=38) (B) and within term birth (n=41) (C). Only the differentially expressed genes found between the inflammatory lesions versus the placenta without lesions within all the patients (group A) showed up-regulated genes which had an over-representation of inflammatory pathways (red) and down-regulated genes have an enrichment of biological pathways (blue) (D).
**Figure S1. Main pathways are over-represented in PTB.** We clustered all of the up-regulated pathways using Cytoscape to remove redundancy (Biological processes, medium network specificity, pathway p<0.05) and notice three major pathways which are up-regulated in PTB namely lymphocyte chemotaxis (white), neutrophil migration (grey), and antibacterial humoral response (black).

**Figure S2. Genes dysregulated in cytokine-cytokine receptor interactions.** Genes up-regulated (red) and down-regulated (green) in KEGG pathway enrichments provide potential targets for future work looking to regulate these pathways.

**Figure S3. Gating strategy to detect proportions of immune cells.** All viable, single cells were divided into CD3+ and CD3- populations based on the CD3 (FITC) marker (A, center). From the CD3- gate, natural killer (NK) cells (CD3-/CD56+) were gated using the CD56 (APC) marker (A, bottom), B lymphocytes (CD3-/CD19+), were gated using the CD19 (V500) marker (B, bottom) and monocytes (CD3-/CD14+), were gated using CD14 (Alexa fluor 700) marker (C, bottom). From the CD3+ gate, NK-T cells (CD3+/CD56+) were gated on using the CD56 (APC) marker (B, center), CD8+ cells were gated on using the CD8 (PerCP.Cy5.5) marker (A, top), and CD4+ cells were gated on using CD4 (APC-Cy7) marker (A, top). From the CD4+ gate, Treg cells (CD3+/CD4+/CD25+) were gated using the CD25 (BV605) marker (B, top), CCR4+ cells were gated using the CCR4 (BV421) marker and CXCR3+ cells were gated on using CXCR3 (PE-CF594) marker (C, top). From CCR4+ cells, Th cell subsets were gated based on their expression of CD4 (APC-Cy7) and high (Th17: CD3+/CD4+/CD194+/CD196^{high}) or low (Th2: CD3+/CD4+/CD194+/CD196^{low}) expression of CCR6 (PE-Cy7) (D, top). Likewise, from
CXCR3+ cells, Th cell subsets were gated based on their expression of CD4 (APC-Cy7) and high (ThTh17: CD3+/CD4+/CD183+/CD196\textsuperscript{high}) or low (Th1: CD3+/CD4+/CD183+/CD196\textsuperscript{low}) expression of CCR6 (PE-Cy7) (D, center). Data were analyzed using FlowJo software.

Figure S4. Proinflammatory mediators have no significant correlation to gestational age. IL-6 levels in term and PTB placentas show correlations to gestational age (T: $R^2=0.104$, $p<0.05$, a PTB: $R^2=0.220$, $p<0.01$, A). Both term and PTB show no significant correlations to gestational age for their expression of placental TNF-α (T: $R^2=0.023$, $p=0.35$, PTB: $R^2=0.006$, $p=0.65$, B). Statistical analysis by simple linear regression.
Table 1

<table>
<thead>
<tr>
<th>Maternal Characteristics</th>
<th>Term (n=41)</th>
<th>PTB (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal Age</strong> (years)</td>
<td>34 (22-42)</td>
<td>31 (18-42)*</td>
</tr>
<tr>
<td><strong>Ethnicity</strong> (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African</td>
<td>8 (19.5)</td>
<td>8 (21)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>21 (51)</td>
<td>28 (73.7)</td>
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<tr>
<td>Other</td>
<td>12 (29.2)</td>
<td>2 (5.2)</td>
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<tr>
<td><strong>BMI</strong></td>
<td>24.6 (18.3-39)</td>
<td>25.6 (17.6-41)</td>
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<tr>
<td><strong>Family history</strong> (HT &amp; DB)</td>
<td>17 (42.5)</td>
<td>14 (39)</td>
</tr>
<tr>
<td><strong>Primiparity</strong> (%)</td>
<td>11 (35.5)</td>
<td>20 (65.5)*</td>
</tr>
<tr>
<td><strong>History of HT/DM (%)</strong></td>
<td>17 (42.5)</td>
<td>15 (40.5)</td>
</tr>
<tr>
<td><strong>GA at delivery</strong> (weeks)</td>
<td>39.8 (38-41.4)</td>
<td>32.4 (26.1-36.7)**</td>
</tr>
<tr>
<td><strong>Birthweight</strong> (grams)</td>
<td>3534 (2850-4440)</td>
<td>1666.5 (680-2685)**</td>
</tr>
<tr>
<td><strong>SGA (%)</strong></td>
<td>0/41 (0)</td>
<td>16/38 (42)**</td>
</tr>
<tr>
<td><strong>HT current pregnancy (%)</strong></td>
<td>0 (0)</td>
<td>2 (5.4)</td>
</tr>
<tr>
<td><strong>DM current pregnancy (%)</strong></td>
<td>4 (9.8)</td>
<td>8 (22)</td>
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<tr>
<td><strong>Induction of Labor (%)</strong></td>
<td>13 (32)</td>
<td>14 (37)</td>
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<tr>
<td><strong>Delivery by CS (%)</strong></td>
<td>25 (60)</td>
<td>20 (52)</td>
</tr>
<tr>
<td><strong>Sex (% of male)</strong></td>
<td>21 (51)</td>
<td>20 (52)</td>
</tr>
</tbody>
</table>

Table 1: Patient obstetrical and demographics data

Body mass index: BMI, Hypertension: HT, Diabetes mellitus: DM, Gestational age: GA, Caesarean delivery: CS, small for gestational age (<10th percentile): SGA Data presented as mean (range) or n (%). *=p<0.05, **=p<0.01, ***=p<0.0001. Statistical analysis by unpaired t-test or Fisher’s exact test where appropriate.
Table 2.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>LogFC</th>
<th>AveExpr</th>
<th>Adj.P.Val</th>
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<td>RPL3P4</td>
<td>Ribosomal protein L3 pseudogene 4</td>
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<td>ENSG00000267954</td>
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<td>ADAM9</td>
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<td>1.35</td>
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<td>PPP1R18</td>
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<td>KCNQ1</td>
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<td>HLA-E</td>
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<td>CPNE7</td>
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<td>2.48x10^{-3}</td>
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<td>DPEP1</td>
<td>Pseudogene dipeptidase 1</td>
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<td>CCL20</td>
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<tr>
<td>TCAF2C</td>
<td>TRPM8 channel associated factor 2C</td>
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<td>OPKR1</td>
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<td>FHDN1</td>
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<td>-1.02</td>
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<td>RSC1A1</td>
<td>Regulator of solute carriers 1</td>
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<td>-2.03</td>
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</tbody>
</table>

Table 2. Top differentially expressed genes between term and preterm deliveries.

Top 10 most up-regulated DEGs in PTB (above) and top 10 most down-regulated DEGs in PTB (below) sorted by logFC (p<0.05). Gene name is represented by Ensembl Gene ID when the gene has not been previously defined. logFC represents log₂Fold changes between preterm and term births, AveExpr represents average log₂Fold change within sequencing and adj.P.Val is the adjusted p-value.
Table S1. Detailed placental histology from term and preterm deliveries.

All placental lesions were analyzed by an expert pathologist according to the international Amsterdam criteria. Data presented as abnormality (%). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. Statistical analysis by unpaired t-test.