Cell type markers indicate distinct contributions of decidual stromal cells and natural killer cells in preeclampsia

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Abstract

Preeclampsia is a devastating pregnancy disorder and a major cause of maternal and perinatal mortality. By combining previous transcriptomic results on preeclampsia with single-cell sequencing (scRNA-seq) data, we here predict distinct and partly unanticipated contributions of decidual stromal cells and uterine natural killer cells in early- and late-onset preeclampsia.

In brief

Preeclampsia is a common serious disorder that can occur during pregnancy. This study uses integrative analysis of preeclampsia transcriptomes and single-cell transcriptomes to predict cell type-specific contributions to preeclampsia.

Preeclampsia is commonly classified as early-onset (EOP) (before week 34) and late-onset preeclampsia (LOP). EOP is characterized by defective placentation and spiral remodeling, whereas in LOP normal placentation has apparently taken place but placental capacity is exceeded and/or other factors contribute to the symptoms. Recent studies suggest that maternal cell types, such as endometrial stromal cells, have more critical role in the etiology of preeclampsia than previously anticipated (Conrad *et al*., 2017; Garrido-Gomez *et al*., 2017). However, the current models of preeclampsia only loosely explain their relative contributions and the cell type-specific molecular mechanisms.

Here we conduct an analysis that combines decidual bulk transcriptomic data from preeclampsia with recently published single-cell sequencing (scRNA-seq) data from healthy women. Specifically, we utilized the previously detected differentially expressed genes for severe EOP and severe LOP from decidua samples from term pregnancies (Tong *et al*., 2018), and from secretory phase of the menstrual cycle of women that previously experienced severe preeclampsia (Garrido-Gomez *et al*., 2021), and used the cell type marker lists from scRNA-seq transcriptomic atlases from endometrium taken during the secretory phase of menstrual cycle (Wang *et al*., 2020), decidua from women undergoing selective pregnancy termination during the 1st trimester (Vento-Tormo *et al*., 2018), and decidua from women at term pregnancy (Pique-Regi *et al*., 2019). With this integrative analysis we were able to predict distinct cell type-specific contributions to the pathogenesis of both EOP and LOP.

We first re-ran the cluster analysis for the three scRNA-seq datasets using Seurat 4.0 (Hao *et al*., 2021), re-annotated the main uterine cell types concordantly (**Fig. 1A, B and C**), identified cell type-specific marker genes and combined these from the three studies to an extended cell type marker list. To search for the presence of cell type-specific markers among the preeclampsia regulated genes, we intersected scRNA-seq marker lists and disease genesets and conducted Fisher's exact tests (**Fig. 1D**). We discovered two robust overrepresentation signals: LOP downregulated genes were enriched with endometrial/decidual stromal cell (dS) markers (combined uterine markers $P = 1.5 \times 10^{-32}$, 5.1-fold) and EOP upregulated genes were enriched with uterine natural killer cell (uNK) markers ($P = 1.3 \times 10^{-22}$, 8.6-fold) (**Fig. 1D**). This suggests that LOP and EOP have distinct cell type-specific etiological characteristics. While LOP associates with transcriptomic changes in stromal cells, EOP is more closely associated with transcriptomic changes in uNKs. As decidualization takes place spontaneously during the non-pregnant menstrual cycle, these results also suggest that the defects in uterine differentiation that contribute to preeclampsia may be detectable already in the non-pregnant menstrual cycle. However, in the analyzed dataset of genes differentially expressed in secretory phase of menstrual cycle from women that previously had preeclampsia (Garrido-Gomez *et al*., 2021), the most significant signature was observed for perivascular cells with less striking enrichment ($P = 2.0 \times 10^{-10}$, 3.7-fold) compared to those we observed with EOP and LOP from (Tong *et al*., 2018).

We next focused on the cell subpopulations among stromal cells in the 1st trimester data (Vento-Tormo *et al*., 2018). The three annotated subpopulations (dS1, dS2, dS3) of stromal cells reflect the stages on the decidualization (differentiation), and dS3 represents decidualized cells with high prolactin expression (Vento-Tormo *et al*., 2018). We repeated the Fisher's exact tests separately for dS1, dS2, and dS3 (**Fig. 1E**), and observed enrichment of dS3 markers among the LOP downregulated genes ($P = 3.7 \times 10^{-38}$, 16-fold). This suggests that in LOP the normal decidualization of maternal stromal cells is defected, further supporting the recent observation by others on severe preeclampsia (Rabaglino *et al*., 2015; Garrido-Gomez *et al*., 2017, 2021). These dS3 - LOP downregulated genes had progressively higher expression during decidualization in healthy donors (**Fig. 1F**), and they were functionally enriched with terms such as "Epithelial Mesenchymal Transition" (EMT) (Hallmark, $P = 6.1 \times 10^{-8}$) and "Wounding" (GO, $P = 5.9 \times 10^{-5}$). To visualize the decidualization trajectory and the expression of selected genes associated with these terms in healthy donors, we used Slingshot (Street *et al*., 2018) (**Fig. 1G,**

H and I). For instance, the expression levels of decorin (*DCN*) and galectin-1 (*LGALS1*) are markedly increased during the decidualization and have the highest expression in dS3 (**Fig. 1H and I**). DCN is a decidualization marker and LGALS1 enhances maternal immunotolerance during pregnancy. On the other hand, LOP upregulated genes were overrepresented with dS1 marker genes ($P = 1.8 \times 10^{-11}$, 9.9-fold) (**Fig. 1E**), including fibroblast marker, actin alpha 2 (*ACTA2*), for the non-decidualized stage, being again in line with the prediction that decidualization defects contribute to LOP. We also observed that the EOP downregulated genes were similarly enriched with dS3 markers ($P = 1.3 \times 10^{-11}$, 7.6-fold), suggesting that gene regulatory changes associated with decidualization defects of stromal cells also contribute to EOP, but not to the same extent as in LOP.

For uNK subpopulations (dNK1, dNK2 dNK3) (Vento-Tormo *et al*., 2018) we did not detect robust subpopulation specific overrepresentation among EOP or LOP regulated genes. We hypothesized that this is because the subpopulation clusters are only moderately separated from each other. Thus, we conducted a functional enrichment analysis of all the uNK cell type marker genes that were upregulated in EOP (**Fig. 1D**) and found terms such as "Allograft rejection" (Hallmark, $P = 4.9 \times 10^{-8}$) and "Leukocyte activation" (GO, $P = 5.1 \times 10^{-8}$) (**Fig. 1J**), which are generally associated with reduced maternal immunotolerance. Although in healthy donors these uNK marker genes did not present as clear differentiation trajectory associated expression patterns as observed in stromal cells, we observed a modest trend of genes in "Allograft rejection" to be more expressed in the dNK2 and dNK3 populations compared to uNK1 (**Fig. 1J**), including for example *CD7* (**Fig. 1K and L**). This suggests that uNK markers upregulated in the more differentiated subpopulations (dNK2 and dNK3) may associate with reduced immunotolerance and preeclampsia.

Finally, for the top overrepresentation gene intersect for women with previous preeclampsia (Garrido-Gomez *et al*., 2021), the perivascular cells – preeclmapsia downregulated signature, we also conducted a functional enrichment analysis. The detected top terms were "EMT" (Hallmark, $P = 4.4 \times 10^{-9}$) and "blood vessel development" (GO, $P = 7.8 \times 10^{-9}$). Notably, EMT term was also detected for the stromal dS3 - LOP downregulated intersect, and the perivascular cell clusters have been associated with endometrial stromal stem cells (Queckbörner *et al*., 2021) that have previously been reported to underlie reproductive disorders.

Main limitations of this study involve the temporal specificity in the dataset intersections. First, EOP and LOP data (Tong *et al*., 2018) were collected upon delivery and the transcriptomic signatures upon delivery may be different from the initial signatures for EOP and LOP earlier during the pregnancy (Rabaglino and Conrad, 2019). Second, we selected to combine the scRNA-seq cell type specific markers from the three different timepoints in order to have more comprehensive cell type marker lists, but this inevitably decreased the temporal specificity of these lists for a given one timepoint.

In conclusion, our analysis with single-cell data derived marker genes extends the resolution of previous heterogenous EOP versus LOP comparisons. We observed that preeclampsia downregulated genes (especially in LOP) are enriched with the markers of decidualized stromal cells (dS3) indicating a critical role for decidualization defects in the etiology of preeclampsia. Our results support previous observations on the importance of decidualization defects (Rabaglino *et al*., 2015; Garrido-Gomez *et al*., 2017, 2021). Notably, these studies collected severe preeclampsia samples without classification to EOP and LOP, whereas our analysis presents a novel observation that the overrepresentation signal for stromal cell decidualization defects is stronger in LOP rather than EOP. Curiously, decidual stromal cells have been suggested to have independently evolved two of their main functions, firstly the anti-inflammatory reaction associated with implantation in the stem of placental mammals, and secondly the ability to support extended maintenance of pregnancy in Euarchontoglires (supraprimates) including humans (Chavan *et al*., 2016). The robust LOP specific signal may be associated with the function of decidual stromal cells to support maintenance of pregnancy rather than the initial anti-inflammatory role in implantation and early placentation. These results, therefore, emphasize the abovementioned dual role of stromal cells in reproductive health. On the other hand, uNK markers linked with reduced immunotolerance were specifically enriched in the EOP upregulated genes which is in line with the critical role of uNK cells to promote immunotolerance during spiral artery modeling and placentation. Overall, our analysis highlights the potential of and motivates future single-cell studies on preeclampsia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this article.

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Author contribution statement

K T R designed the work. K T R, N A and M M performed analysis. K T R, N A, M M, T L, M P and L L E wrote the paper.

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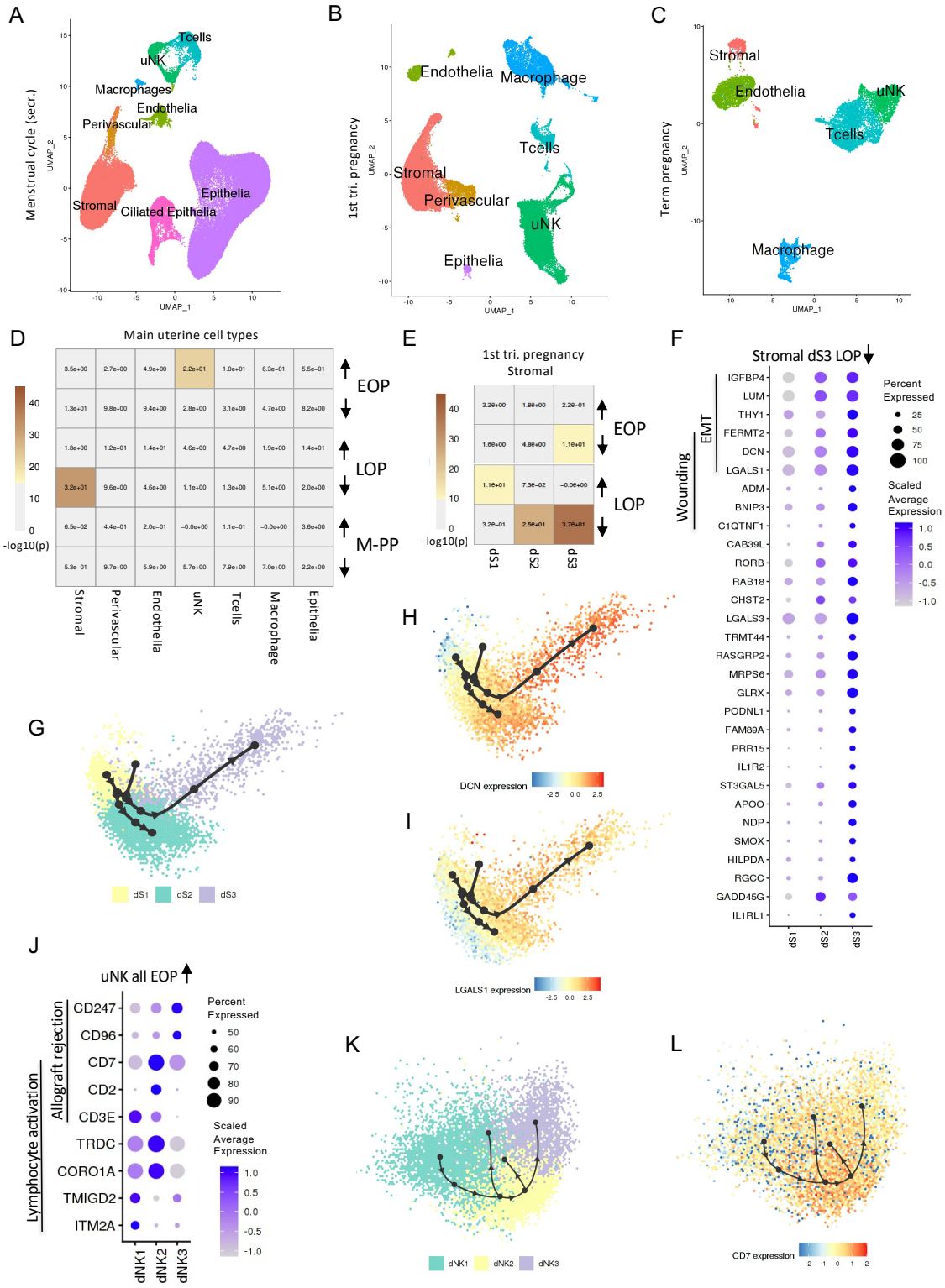
Figure Legend

Figure 1. Preeclampsia regulated genes are enriched with decidual stromal cell and uterine natural killer cell marker genes. The uterine cell types in (A) secretory phase menstrual cycle (Wang *et al*., 2020), (B) 1st trimester pregnancy (Vento-Tormo *et al*., 2018), and (C) term not in labor pregnancy (Pique-Regi *et al*., 2019) single-cell transcriptomes with harmonized annotation. For (A) and (B) the preprocessed scRNA-seq UMI counts were extracted from ArrayExpress accession E-MTAB-6701 (Vento-Tormo *et al*., 2018) including 6 decidua samples (6-12 weeks of gestation) with a total of 36,186 cells and GEO accession GSE111976 (Wang *et al*., 2020) including 10 endometrial samples (cycle days 16-26) with a total of 71,032 cells,

207 respectively. For (C) the raw data was extracted from dbGaP accession phs001886.v1.p1
208 (Pique-Regi *et al*., 2019) with consent, only term no labor (basal plate, chorioamniotic mem-
209 branes) samples were selected with total of 13,730 cells. All the three datasets were produced
210 using 10X Genomics scRNA-seq. Samples were pre-processed using cellranger-3.1.0's count
211 function with default parameters and prebuilt reference genome (hg38). We used the standard
212 analysis protocol of Seurat 4.0 (Hao *et al*., 2021) and the annotation of the clusters was harmo-
213 nized to follow the one from the 1st trimester study (Vento-Tormo *et al*., 2018). (D) A heatmap
214 of Fisher's exact test p-values for the overlaps of uterine cell type marker genes and preeclamp-
215 sia differentially expressed genes. The arrows mark the subsets that are up- or downregulated
216 genes in the original studies. The marker gene lists used for each main cell type were combined
217 lists from the condition specific cell type markers from A, B and C. Early-onset (before week
218 34) severe preeclampsia (EOP) (n = 3), late-onset severe preeclampsia (LOP) (n = 3) and con-
219 trol (n = 3) samples were from term deciduas delivered by cesarean section (Tong *et al*., 2018).
220 Samples for women with previous severe preeclampsia (M-PP) (n = 17) together with controls
221 (n = 12) were from late secretory menstrual cycle (days 22-32) endometrium (Garrido-Gomez
222 *et al*., 2021). Statistical significance of the enrichment was determined using Fisher's exact test
223 in R, with all Refseq protein-coding genes (n = 20203) as the background. (E) A heatmap of
224 Fisher's exact test p-values for the overlaps of the preeclampsia differentially expressed genes
225 and the subpopulations of endometrial/decidual stromal cells (dS1, dS2, dS3) from the 1st tri-
226 mester study. (F) Genes in the LOP downregulated - stromal dS3 marker intersection (1st
227 trimester). (G) Trajectory inference of differentiating dS cells using Slingshot (Street *et al*.,
228 2018). Slingshot uses minimum spanning tree algorithm to reconstruct the lineage structure
229 among differentiating cells from scRNA-seq data. In the 2D visualization the axes represent
230 the first principal components. The black dots represent the cells in the lower dimension and
231 the arrow-headed lines depict the suggested differentiation trajectories estimated by Slingshot.
232 (H) Decorin (*DCN*) and (I) galectin-1 (*LGALS1*) expression across the dS cell trajectories. (J)
233 Genes in the EOP upregulated - uNK cell type marker intersection. (K) Trajectory inference of
234 uNK cell subpopulations from the 1st trimester study using Slingshot. (L) *CD7* expression
235 across the uNK trajectories. For (E-L) only the 1st trimester data was used. For (F) and (J) the
236 top functional enrichment categories (GO, Hallmark) are displayed on the left. Functional
237 enrichment analysis was done using the Metascape tool with all available pathway databases
238 (GO Biological Processes, Reactome Gene Sets, Canonical Pathways, Biocarta Gene Sets,
239 WikiPathways, KEGG Pathways, and Hallmark Gene Sets). Trajectory inference was done

using the infer_trajectory function of Slingshot in the “dyno” version '0.1.2' (Saelens *et al*., 2019).


A
Menstrual cycle (secr.)
Tcells
uNK
Macrophages
Endothelia
Perivascular
Stromal
Ciliated Epithelia
Epithelia
UMAP_1
UMAP_2
B
1st tri. pregnancy
Endothelia
Macrophage
Tcells
Stromal
Perivascular
uNK
Epithelia
C
Term pregnancy
Stromal
Endothelia
uNK
Tcells
Macrophage
D
Main uterine cell types
-log10(p)
EOP
LOP
M-PP
Stromal
Perivascular
Endothelia
uNK
Tcells
Macrophage
Epithelia
E
1st tri. pregnancy
Stromal
-log10(p)
EOP
LOP
dS1
dS2
dS3
F
Stromal dS3 LOP
Wounding
EMT
IGFBP4
LUM
THY1
FERMT2
DCN
LGALS1
ADM
BNIP3
C1QTNF1
CAB39L
RORB
RAB18
CHST2
LGALS3
TRMT44
RASGRP2
MRPS6
GLRX
PODNL1
FAM89A
PRR15
IL1R2
ST3GAL5
APOO
NDP
SMOX
HILPDA
RGCC
GADD45G
IL1RL1
1sp
2sp
3sp
Percent Expressed
Scaled Average Expression
G
dS1
dS2
dS3
H
DCN expression
I
LGALS1 expression
J
uNK all EOP
Lymphocyte activation
Allograft rejection
CD247
CD96
CD7
CD2
CD3E
TRDC
CORO1A
TMIGD2
ITM2A
dNK1
dNK2
dNK3
Percent Expressed
Scaled Average Expression
K
dNK1
dNK2
dNK3
L
CD7 expression