1 **Single-cell immune landscape of human recurrent** 2 **spontaneous abortion**

3
4 Feiyang Wang^{1,4,#}, Wentong Jia^{1,4,#}, Mengjie Fan^{2,#}, Zhilang Li^{1,4}, Yongjie
5 Liu^{3,4}, Yeling Ma^{1,4}, Xuan Shao^{1,4}, Yu-xia Li¹, Rong Li^{2,*}, Qiang Tu^{3,4,*}, Yan-Ling
6 Wang^{1,4,5,*}

7
8 ^{1.} State Key Laboratory of Stem Cell and Reproductive Biology, Institute of
9 Zoology, Chinese Academy of Sciences, Beijing 100101, China

10 ^{2.} National Clinical Center for Obstetrics and Gynecology, Peking University
11 Third Hospital, Beijing 100191, China

12 ^{3.} State Key Laboratory of Molecular Developmental Biology, Institute of
13 Genetics and Developmental Biology, Innovation Academy for Seed Design,
14 Chinese Academy of Sciences, Beijing 100101, China

15 ^{4.} University of Chinese Academy of Sciences, Beijing 101408, China

16 ^{5.} Lead contact

17 [#], These authors contribute equally to this work

18 ^{*}, All correspondence should be addressed to Dr. Yan-Ling Wang at
19 wangyl@ioz.ac.cn, or Dr. Qiang Tu at qtu@genetics.ac.cn, or Dr. Rong Li at
20 roseli001@sina.com

21

22

SUMMARY

Successful pregnancy in placental mammals substantially depends on the establishment of maternal immune tolerance to the semi-allogenic fetus. Disorders in this process are tightly associated with adverse pregnancy outcomes including recurrent spontaneous abortion (RSA). However, an in-depth understanding of the disorders from the aspect of systematic and decidual immune environment in RSA remains largely lacking. In this study, we utilized single-cell RNA-sequencing to comparably analyze the cellular and molecular signatures of decidual and peripheral leukocytes in normal and RSA pregnancies at the early stage of gestation. Integrative analysis identified 22 distinct cell clusters in total, and a dramatic difference in leukocyte subsets and molecular properties in RSA cases was revealed. Specifically, the cytotoxic properties of CD8T effector, NK, and MAIT cells in peripheral blood indicated apparently enhanced immune inflammatory status, and the subpopulation proportions and ligand-receptor interactions of the decidual leukocyte subsets demonstrated preferential immune activation in RSA patients. The molecular features, spatial distribution and the developmental trajectories of five decidual NK (dNK) subsets were illustrated. The proportion of a dNK subset responsible for fetal protection was reduced, while the ratio of another dNK subset with cytotoxic and immune-active signature was significantly increased. Notably, a unique pro-inflammatory CD56+CD16+ dNK subpopulation was substantially accumulated in RSA decidua. These findings reveal a comprehensive cellular

45 and molecular atlas of decidual and peripheral leukocytes in human early
46 pregnancy, which provides an in-depth insight into the immune pathogenesis
47 for early pregnancy loss.

48

49 **KEY WORDS:** Decidual and peripheral leukocytes, single-cell RNA-seq, early
50 pregnancy, developmental trajectory

51

52

INTRODUCTION

Reproductive success in placental mammals substantially depends on the establishment of maternal immune tolerance to the semi-allogenic fetus during pregnancy (Ander et al., 2019; Erlebacher, 2013). In humans, failures in immune tolerance are tightly associated with various adverse pregnancy outcomes, mainly including recurrent spontaneous abortion (RSA), preeclampsia, and stillbirth (Deshmukh and Way, 2019; Fisher, 2015; Kheshtchin et al., 2010)

The key of fine-tuned immune tolerance at the feto-maternal interface is the well-coordinated crosstalk among uterine mucosal immune cells and extraembryonic placental cells. The maternal immune cells populating the uterine mucosa include decidual NK (dNK) cells (~50-70%), macrophages (~20%), T cells (~10-20%) and a small amount of DC and B cells. The numerically dominant dNK cells are characterized by low cytotoxicity and strong cytokine producing capacity, which is divergent from peripheral NK (pNK) cells (Wang et al., 2014). Mechanistically, the inhibitory receptors LILRB2 and KIR2DL4 on dNK cells recognize HLA-G on the surface of trophoblast cells, leading to the suppressed cytotoxic capacity of dNK cells (Gamliel et al., 2018a; Parham and Moffett, 2013). Moreover, the biased differentiation of CD4⁺ T cells towards Th2 and Treg has been confirmed vital to the tolerance of fetal cells in decidua (Wang et al., 2010; Yang et al., 2008).

Murine decidual immune cells, especially innate lymphocytes, have been

75 characterized as multiple subsets that present dynamical lineage
 76 differentiation and crosstalk properties during pregnancy (PrabhuDas et al.,
 77 2015). However, the corresponding systematic study on human decidua is
 78 largely lacking, which hampers the in-depth understanding of the
 79 physio-pathological regulation of human pregnancy. A recent paradigm shifting
 80 study by Moffett and colleagues utilizing single-cell RNA-sequencing and
 81 comprehensive data analysis constructed a detailed cellular map and the
 82 elaborate cell-cell communication patterns of the human decidual-placental
 83 interface. Specifically, three major subsets of dNK cells that have distinctive
 84 immunomodulatory and chemokine profiles were identified (Huhn et al., 2020;
 85 Suryawanshi et al., 2018; Vento-Tormo et al., 2018). However, high-resolution
 86 immune landscape of dysregulated decidua in the contexts of pregnancy
 87 complications, for example in RSA, is still unknown, which greatly limit the
 88 exploration of immune-related pathogenesis for recurrent miscarriage.

89 In the present study, we utilized single-cell RNA-sequencing to comparably
 90 analyze the cellular and molecular signatures of decidual and peripheral
 91 leukocytes in normal and RSA pregnancies at the early stage of gestation. By
 92 integrating the gene expression properties, ligand-receptor interactions, and
 93 spatial localization patterns, the cell-type-specific communications among
 94 various subsets of leukocyte and the developmental trajectory of dNK cells
 95 were constructed. A pathological leukocyte atlas in RSA decidua and
 96 peripheral blood was illustrated. Our findings reveal a detailed molecular and

cellular map of decidual and peripheral leukocytes in early pregnancy of human, and highlight the integral harmful immune responses that may lead to early pregnancy failure.

RESULTS

Clustering of immune cells from peripheral blood and decidual tissues in normal and RSA pregnancies

To characterize the immune cells in RSA, we applied scRNA-seq to study CD45+ cells isolated from peripheral blood and decidual tissues of three pairs of normal pregnant women and RSA patients with unknown cause. Viable CD45+ leukocytes were enriched by FACS and subjected to droplet-based single-cell RNA sequencing (scRNA-seq) using the 10x Genomics Chromium platform (Fig. S1A and B). Sequencing data from six peripheral blood samples and six decidual tissue samples were obtained from 12 scRNA-seq libraries. Following computational quality control and filtering using the Seurat package (Butler et al., 2018; Stuart et al., 2019), the final datasets containing 56,758 high-quality cells were subjected to further analysis (Fig. S1C, D and E).

We performed an unsupervised graph-based clustering to analyze scRNA-seq data in Seurat (version 3.0.3). In order to eliminate unreasonable clustering due to batch effects, we integrated the data using the reciprocal PCA function of Seurat. Overall, 22 transcriptionally distinct clusters were identified

(Fig. 1A, B and C), and were further confirmed using the MNN algorithm (Haghverdi et al., 2018)(Supplementary Fig. S2). The cell identity of the 22 clusters was assigned on the basis of known marker genes and literature evidence (Vento-Tormo et al., 2018)(Suryawanshi et al., 2018) (Fig. 1D).

In peripheral blood, T cells were identified and grouped into five major clusters, including two subsets of Naïve T cells (CD4T Naive and CD8T Naive) which express the marker gene *CCR7*, mucosal-associated invariant T cells (MAIT) specifically expressing *ZBTB16*, CD4 Memory T cells (CD4T Memory) annotated by *S100A4*, and CD8 T effector cells (CD8T effector) that express marker genes *FCGR3A* and *FGFBP2* (Fig. 1D; Supplementary FigS3). Peripheral NK cells were clustered as CD56dimCD16+ NK cells (NK dim) and CD56brightCD16- NK cells (NK bright). Monocyte included CD14+ subset and CD16+ subset. In addition, a low abundance of B cells (identified by *CD79A* expression) and DC cells (annotated by *LYZ* and *FCER1A*) were captured in peripheral blood.

In decidual tissues, the most abundant population appeared as dNK cells, which were subgrouped into five clusters, named as dNKa, dNKb, dNKc, dNKd, and dNKe. The dNKe subset presented positive expression for *FCGR3A*/CD16, which was uniquely accumulated in RSA decidual tissues. Decidual T cells were clustered as CD4+, CD8+, and a relatively small number of FOXP3+ regulatory T cells (dCD4T, dCD8T, and dTreg). Other decidual leukocyte

clusters included *KIT*-expressing ILC3 cells (ILC3), *CD14*-expressing macrophage (dM), *FCER1A*-expressing dendritic cells (DC), and *TPSAB1*-marked mast cells (Mast) (Fig. 1A and D).

Cellular and molecular characteristics of the peripheral leukocytes indicate a systematic pro-inflammatory feature in RSA patients

Given T cells present a dominant proportion in peripheral leukocytes, we therefore first analyzed the properties of T cell clusters in RSA and normal pregnancies. The gene expression profiles of the five peripheral T cells subsets were indistinguishable between RSA and normal pregnancies (Fig. 2A). However, the proportion of each T cell cluster exhibited evident alterations in RSA patients, presenting by reduced CD4 T Naïve, CD8 T Naïve and CD4 T Memory populations, and augmented CD8 T effector and MAIT populations (Fig. 2B). In addition, transcription of inflammatory factors, such as *IRF1*, *RORA* in MAIT cells and *IFNG* in CD8 T effector cells and what was remarkably increased in RSA cases (Fig. 2C and D).

The other significant change in RSA peripheral leukocytes was the increased proportion of CD56dimCD16+ NK dim cells (Fig. 2E). Furthermore, in these cells, evidently decreased expression of immunosuppressive genes, such as *TIGIT*, *KLRG1*, *IL32* and *ALOX5AP*, while increase in pro-inflammation genes including *HLA-B*, *NR4A2*, *JUN*, *JUNB*, *NFKBIA* and

MAP3K8 were observed in RSA cases (Fig. 2F). Take together, the properties of the peripheral leukocytes, including aforementioned cell composition shift and specific gene expression pattern in certain cell cluster, strongly indicate an overall enhanced systematic pro-inflammatory immune feature of RSA patients.

Cellular atlas of the decidual leukocytes in RSA pregnancy

Increasing evidence has demonstrated compromised immune cell differentiation or function in RSA decidua. However, a global cellular change remains largely undefined. Our analysis of cell subset proportion in decidua clearly revealed an increased portion of dCD8 T, dNKd, and dNKe populations, and lowered ratio of dNKa, dNKc, and dM cells in RSA decidua (Fig. 3A). To systematically investigate the cell-cell communications among these cell subsets, we analyzed the expression levels of immune cell relevant ligand-receptor interacting pairs within cell types (Fig. 3B and C). Significantly increased expression of *IFNG* and *TNF* was found in dCD8 T, ILC3 and dM in RSA decidua, which may account for the accumulation of dNKe or dCD4T cells that exhibited remarkably higher expression of the corresponding receptors for these two cytokines. The receptor for *CXCL16*, i.e. *CXCR6*, was predominantly expressed in dCD4T and dCD8T. Therefore, the accumulation of T cell subsets in RSA decidua may attribute to the enhanced *CXCL16* in dM. In addition, the expression of *CCL3*, *CCL4*, and *CCL5* in different immune cell subsets also

reflected the activation of the immune micro-environment at the maternal-fetal interface and the vigorous recruitment of macrophages (Du et al., 2014). Such molecular interactions among cell populations via specific ligand-receptor complexes indicated a potential mechanism of immune activation that caused compromised immune environment in RSA decidua.

Differential gene expression profiles in decidual macrophage of RSA pregnancy

Alteration in global gene expression profiles in the RSA leukocyte subset was typically observed in dM. As shown in the volcano plot, using fold-change (FC) >1 and p-values <0.05 as cutoffs, 96 upregulated genes and 27 downregulated genes were identified in RSA dM relative to its normal counterpart (Fig. 3D).

Decidual macrophages mediate multifunctional immunoregulation, such as antigen presentation, phagocytosis, recruitment of other immune cells (Varol et al., 2015)(Gentek et al., 2014). The increased expression of *CXCL8*, *TNF*, and *IFIT2* genes in RSA dM indicated the impaired immune protection. Moreover, the suppressed level of angiogenic factor *VEGFA* and the immunosuppressive molecule *LAGLAS1*, while enhanced expression of pro-inflammatory factors *CXCL8* and *TNF* in RSA dM cell were observed (Fig. 3E). These data indicated a preferentially pro-inflammatory property of dM in

RSA patients.

Molecular aspects and developmental trajectories of human dNK subtypes

Consistent with the findings in previous literatures (Bulmer et al., 1991; King et al., 1991), dNK cells accounted for more than 70% of total decidual leukocytes in our dataset. The characteristics of the five dNK subpopulations were further illustrated by the top 20 marker genes (Fig. 4A) and KEGG analysis (Fig. 4B). As shown, dNKa cells highly expressed *MCM5*, *STMN1*, and *PCNA*, indicating their manifest proliferative capacity. The defining markers for dNKb cells included *XCL1*, *XCL2*, and *ZNF683*. dNKc cells strongly expressed *ENTPD1* (CD39), *CSF1* and *PKM*, and showed active oxidative phosphorylation and glycolysis activity. dNKd cells were defined by *ITGAE* (CD103), *CD160*, and *KLRB1*, and exhibited certain cytotoxic and immune-activated property. *FCGR3A/CD16*, *CXCR4*, *PLAC8* were enriched in dNKe cells, which indicates an active cytotoxicity status of these cells.

It has been well known that dNK cells possess various receptors for recognition of HLA-C, HLA-G and HLA-E molecules in placental trophoblast cells, which primarily mediates dNK-trophoblast communications. We found that dNKc cells expressed much high level of *KIR2DL3* and *KIR2DL1* which

are inhibitory KIR receptor for HLA-C, and *LILRB1*, the high-affinity receptor for HLA-G. dNKc and dNKb cells also moderately expressed *KLRC2/NKG2C*, *KLRC3/NKG2E* and *KLRC1/NKG2A*, which are receptors for HLA-E. dNKd cells possessed much lower expression of all these receptors. Moderate levels of *LILRB1*, *KLRC2/NKG2C* and *KLRC3/NKG2E* were observed in dNKe cells (Fig. 4C).

To determine whether the dNK subtypes exhibit cell type dependent localization pattern at the feto-maternal interface, we performed immunofluorescent staining for CD56, *ENTPD1*(CD39), *ITGAE*(CD103), CD16 and PLAC8 on serial sections of decidual tissues. Immunohistochemistry for cytokeratin 7 (CK7) was used to locate trophoblast and uterine gland epithelium in the sections. The results showed that CD39 positive dNKc cells were more abundant in decidual compacta where trophoblasts infiltrate, while CD103-labelled dNKd cells mainly distributed in distal compacta through spongiosa, but less in proximal compacta (Fig. 5A, B and C). A very small number of CD16-expressing dNKe cells could be found in decidual compacta, usually near uterine spiral artery (Fig. 5D). These results predict the potential of dNKc cells in the recognition and protection of trophoblasts, and dNKd cells in maintaining certain cytotoxic and immune-active environment in decidua.

Interestingly, apart from dNKe, the other four dNK subtypes co-expressed the tissue-resident markers *CD49a/ITGA1*, indicating their uterine resident

feature. By far the developmental route of uterine resident dNK cells remains unclear. Our result of flow cytometry showed dNKc cells as CD27-CD11b- (Fig. 4D), which indicates their immature developmental status according to the previous report (Fu et al., 2011). Therefore, we further analyzed the developmental trajectory of dNK cells by carrying out pseudotime analysis on our scRNA-seq data. The top 100 genes in each dNK subtype were clustered (Fig. S4), and Monocle2 was performed to infer their development trajectory (Fig. 4E). As shown, dNKa appeared at the very initiation of the trajectory, and dNKc emerge at the early developmental stage with much higher expression of dNK-classical transcription factors *EOMES* and *ID3*. It tended to differentiate towards dNKb and finally dNKd (Fig. 4F). Distance between dNKd and dNKe indicated the probably alternative origin of CD49a-CD16+ dNKe cells.

Alterations of dNK subsets in decidua of RSA pregnancy

To verify the feature of dNK subset proportion in RSA decidua identified by scRNA-seq in Fig 3A, we analyzed dNK cell population by flow cytometry in a larger sample cohort of normal and RSA decidual tissues. We sorted cells by using CD56 (expressed by all dNK cells) and CD3 (negative sorting marker), combined with markers for dNKc (CD39), dNKd (CD103), and dNKe (CD16) that were identified from our scRNA-seq data (Fig. 6A). As indeed, the cell portion of dNKc was uniformly and remarkably reduced by around 50%,

whereas dNKd proportion increased by 1-fold in RSA decidua, compared to the normal pregnancy controls (Fig. 6B). The ratio of dNKe cells was rather small in normal decidua, accounting for less than 4% of total dNK. However, in approximately half of the enrolled RSA cases, dNKe cell population increased to about 12% of total NK cells (Fig. 6B). Consistently, immunofluorescent staining for CD16 in RSA decidua showed evidently more dNKe cells in decidua compacta niche close to blood vessels (Fig. 5D).

Comparison of the differential gene expression in dNK subsets between RSA and normal pregnancy demonstrated a significant increased expression of inflammation-related genes such as *CD69* and *IRF9* in dNKd cells, *INFG*, *CXCL8*, *TNFRSF8*, and *IFIT3* in dNKe cells, as well as downregulation of anti-inflammation genes including *SPINK2* and *AREG* in dNKc cells from RSA patients (Fig. 6C).

DISCUSSION

Cellular and molecular mechanisms accounting for maternal immune tolerance to protect growth of semi-allogenic fetus in placental mammals has been a fascinating scientific question. Increasing evidence demonstrate the significance of the complicated and precisely controlled cell-cell communications within the feto-maternal interface in concert with the well-coordinated systematic immune adaptations along gestation (Ander et al., 2019; Arck and Hecher, 2013; Erlebacher, 2013; Hanna et al., 2006; Nancy et al., 2012). Recently, a molecular and cellular map of normal feto-maternal interface at human early gestation strikingly deepened our understanding of the cell subtypes and their predicted interactions in normal homeostatic decidua (Vento-Tormo et al., 2018). However, the corresponding landscape and dynamics of immune cell at single cell level in failed pregnancy, such as RSA, is lacking to date. To our knowledge, our study is the first comprehensive single-cell transcriptomics atlas of the decidual and peripheral immune cells in human recurrent miscarriage at early gestation. Our findings integrate complex information of immune cell, including cell composition, functional status, developmental trajectory, and predicted cellular interactions among immune cells. Our work illustrates an integral framework of the compromised immune environment that is harmful to the intra-uterine fetus in RSA pregnancy.

The immuno-mechanism for RSA, especially those without fetal

310 chromosomal or congenital abnormalities or other known pathological causes,
 311 has been largely debatable. Multiple studies demonstrated abnormal
 312 expressions or functions of various cytokines, or dysregulated proportions or
 313 properties of certain immune cells in patients with recurrent miscarriage.
 314 However, studies solely focusing on certain limited cell types may result in
 315 misleading points or contradictory results. To date the strategies of early
 316 diagnosis or intervention of RSA are lacking, which makes it hard to reduce the
 317 threat to the patients (Seshadri and Sunkara, 2014). Here by taking
 318 advantage of single-cell sequencing technology, we construct an overall
 319 pathological change in the properties of immune cell subsets in peripheral
 320 blood, which may pave the path for the pre-symptom diagnosis of the
 321 disordered pregnancy. The significantly enhanced differentiation of naive T
 322 cells to CD8T effector cells, and preferential increasing in number of NK dim
 323 cells, together with the upregulated inflammation-related genes, demonstrate a
 324 systematic pro-inflammatory status in RSA patients. These results are
 325 consistent with previous reports (Ebina et al., 2017; Kuon et al., 2017). An
 326 interesting finding is the increased frequency of MAIT cells in RSA peripheral
 327 blood. As a non-conventional T cell subset, MAIT cells are mainly activated by
 328 exposure to microbes, while they can also be turned on by inflammatory stimuli
 329 in the absence of TCR-mediated antigen recognition. There is considerable
 330 evidence to suggest their involvement in a broad range of infectious and
 331 non-infectious diseases (Godfrey et al., 2019). Studies in liver disorders

suggest that the MAIT cells may play a protective role against bacterial infections in a normal liver, but might be detrimental, with over-inflammation, in liver diseases (Bertrand and Lehuen, 2019). Notably, MAIT cells express several cytokine receptors, including IL-1R, IL-7R, IL-12R, IL-15R, IL-18R and IL-23R, and response to these multiple cytokines stimulation (Godfrey et al., 2019). Our sequencing data showed the evidently enhanced expression of *IRF1* and *RORA* in MAIT from RSA periphery, indicating their highly immune-activated property. By far evidence of MAIT cell in pregnant condition has been very limited, and our study for the first time indicate the association of MAIT activation with the maternal inflammation condition that damage fetal survival. Whether the change of MAIT activation is the cause or result of miscarriage remains to be established.

At the maternal-fetal interface, decidual NK cells assemble the largest population of decidual leukocytes at early pregnancy. Unlike their peripheral counterparts, dNK cells are less cytotoxic but actively secret a vast array of factors and cytokines. They have been found to play powerful roles including facilitating the remodeling of uterine spiral arteries, promoting trophoblast invasion and fetal growth, regulating T cell differentiation, increasing the availability of maternal blood at the implantation site, and so on (Ander et al., 2019). In unexplained infertile patients, substantially fewer uterine NK cells were observed when compared to fertile controls (Klentzeris et al., 1994).

However, discrepant results have been reported regarding the change of dNK cell number in RSA patients (King et al., 2010; Seshadri and Sunkara, 2014). Our study clearly demonstrates the abnormal properties of dNK subsets in RSA decidua, including cell composition and gene expression, which suggests impaired regulation of dNK subset development in the patients.

The characteristics of dNKc cells are similar to the highly active dNK1 subset in Vento-Tormo's study. They are numerically dominant dNK cells and represent the typical dNK functions to support embryo growth. The CD11b-CD27- property of dNKc manifests their immature characteristic and the potential to differentiate (Fu et al., 2011). Specific expression of PBX1 was found in CD27-CD11b- dNK cells, and a recent study demonstrated the association of decreased PBX1 expression or PBX1^{G21S} mutation with unexplained RSA (Zhou et al., 2020), further suggesting the crucial role of this dNK subset in pregnancy maintenance. Interestingly, a recently identified pregnancy trained dNK (PTdNK) share similar features with dNKc or dNK1 (Gamliel et al., 2018b), and this subset has been speculated to be enriched and boost decidua receptivity in subsequent pregnancy, therefore may be responsible for the "memory" of reproductive outcomes in next pregnancies. Thus, the strikingly lowered frequency of this subset in RSA patients can partly explain why failed pregnancies repeatedly occur in the patients. In addition to the decreased proportion, the altered gene expression in dNKc of RSA decidua further indicates their diminished immune-protective capability.

376

377 The development trajectory of dNK subsets reveals dNKd cells are
378 relatively mature and immune-activated. They are likely to maintain certain
379 cytotoxic and immune-active environment in decidua, which may contribute to
380 the appropriate extent of trophoblast invasion. However, in RSA decidua their
381 portion raises to more than one fold of the normal counterpart. Besides, they
382 exert enhanced production of pro-inflammatory cytokines, which is likely
383 causing the decidual environment harmful to the fetal cells and other maternal
384 immune cells such as T cells and macrophages (Fridman et al., 2012; Fu et al.,
385 2013; Ma et al., 2017; Sotnikova et al., 2014; Yang et al., 2019).

386

387 Another notable dNK subset in our study is the newly-identified
388 CD56+CD16+ dNKe, which is largely enriched in RSA decidua. Being different
389 from all the other four subsets, dNKe cells do not exert expression of the
390 tissue-resident marker CD49a. Consistently, the developmental trajectory
391 show separation of dNKe from the maturation route of other dNK subsets. It is
392 therefore likely that this subset may origin from other sources. Currently there
393 are three hypotheses for the origin of dNK cells, i.e., 1) recruitment of
394 peripheral blood NK cells to decidua(Carlino et al., 2008), 2) maturation of
395 uterine-resident NK cells in response to IL15 or progesterone(Manaster et al.,
396 2008), 3) direct differentiation from hematopoietic precursors in the decidua
397 upon stimulation of specific decidual factors(Vacca et al., 2011). Considering

the common CD49a-CD16+ feature of dNKe and peripheral NK dim, we further compared the gene expression pattern between dNKe and NK dim. As shown in the volcano plot, using fold-change (FC) >1 and p-values <0.05 as cutoffs, 187 upregulated genes and 73 downregulated genes were identified in dNKe relative to NK dim. However, there are 699 co-expressed genes between dNKe and NK dim, which present their common feature in exercising common cellular functions (Fig. S5). Thus, it is likely that peripheral NK dim cells are recruited to decidua and further educated towards dNKe by some decidual factors. Many studies have demonstrated the crucial role of IFN-gama, CCL3/MIP-1, CXCL10/IP-10, CXCL12/SDF-1 in enrolling pNK to decidua (Hanna et al., 2003; Wallace et al., 2013; Wu et al., 2005). What's more, an in vitro study reveals that human peripheral CD16+ NK cells can be converted to a dNK-like phenotype upon the stimulation of hypoxia, TGF-beta, and the demethylating agent Aza (Zhang et al., 2016). Here our data reveals the evident increase in *INFG* in dCD8T, *CXCL16*, *CCL3* and *TGFB* in dM of RSA decidua, which may be responsible for enrolling and educating excessive pNK cells to decidua compartment. The much higher expression of several pro-inflammatory factors in RSA dNKe cells also indicate their enhanced cytotoxicity in the patients. An interesting previous study demonstrated the extravagant enrichment of CD16+ NK cells in endometrium of RSA patients during their pre-pregnant period (Lachapelle et al., 1996). Therefore, we propose that the patients with more abundant dNKe cells may suffer a greater

chance of pregnancy failure in their subsequent pregnancies.

Decidual macrophages account for around 20% of leukocytes at the
feto-maternal interface, and they have many diverse functions during
pregnancy. Here a relatively large amount of differentially expressed genes
was identified in RSA decidual macrophages, which potentially suggest some
functional abnormalities. For instance, the obviously repressed *VEGFA* in RSA
dM indicates the link to impaired remodeling of the spiral arteries and
angiogenesis. The upregulated genes include immunoinflammatory factors
and the relevant signaling molecules, such as *CXCL8*, *TNF*, *IFIT2*, *JUN*, *JUNB*,
etc., predicting the diminishment in the anti-inflammatory capacity.
Furthermore, the increased expression of IL-8 in dM and enhanced *IFNG*
expression in dNKe of RSA patients are likely in tight correlation, because it
has been demonstrated that IL-8 from dM enhances the production of
IFN-gama in dNK cells (Baratin et al., 2005; Dalbeth et al., 2004). In addition,
our brief repository of ligand-receptor complexes indicated the potential of dM
in recruiting dCD8T or dNKe through *CXCL16-CXCR6* or *TNF-TNFRSF1B*
interactions. Although decidual macrophages are believed to exist
predominantly in a regulatory/homeostatic M2-like (dM2) phenotype, while less
in pro-inflammatory M1 (dM1) phenotype during pregnancy(Erlebacher, 2013),
we did not find an alteration in the proportion of dM1 and dM2 in RSA patients
(Fig. S6). This is probably due to the relatively small amount of captured dM

cells for sequencing.

It has to be noticed that the subset analysis on some low-abundant cell subpopulations, such as decidual T cells, DC cells and macrophages, remains insufficient. Therefore, it warrants further investigation on the subset characterization of these cells by specifically improved enrichment strategy, which will provide a more comprehensive understanding of the complicated cell-cell communication network at the feto-maternal interface. In addition, the analysis of endometrial cell atlas in the pre-pregnant stage of RSA patients will be helpful to find prospective intervention targets and improve their pregnancy success.

In general, our study comprehensively illustrates the compromised immune response in periphery and feto-maternal interface of RSA patients. The findings generate a data-driven hypothesis about immune-related pathogenesis for recurrent miscarriage, and provide new insight into the strategies for diagnosis and intervention of the diseases.

MATERIALS AND METHODS

Sample Collection and Ethic permission

Clinical samples of anti-coagulant peripheral blood and decidual tissues from normal pregnancy (N=10) or recurrent spontaneous abortions (RSA) (N=14) at gestational week 6-8 were obtained upon therapeutic termination of pregnancy at Peking University Third Hospital. The decidual tissues were immersed in iced RPMI-1640 medium and the blood samples were kept on ice. All samples were subjected to cell isolation or fixation within 1 hour following the surgery. The collection of human samples was permitted by the Local Ethical Committees in Peking University Third Hospital.

RSA was defined according to the criteria of Practice Committee of the American Society for Reproductive Medicine. In brief, these patients had history of two or more failed pregnancies with unknown cause (Committee and Society, 2013). Women who manifest endocrine disorder, fetal chromosomal or congenital abnormalities, uterine anatomical disorders, renal disease, or pregnancies conceived by fertility treatment were excluded from this study. All the enrolled patients had arrested fetal development for less than one week before the termination of pregnancy.

Cell isolation and purification

Freshly collected human decidual tissues were trimmed into 1 mm³ piece by GentleMACS Dissociator (Miltenyi Biotec, 130-093-235, Germany) and

digested twice for 30 minutes each at 37°C with 1.0 mg/ml type IV collagenase (Gibco, 9001121, Grand Island, NY, USA) and 10U/ml type I DNase (Sigma, DN25, St Louis, MO, USA). The cell suspensions were filtered through 60 mesh and 200 mesh sieves, and were collected by centrifuging at 1000rpm for 10 min. The resuspended cells were subjected to lymphocyte enrichment using Ficoll-Paque Plus (17-1440-02, GE Healthcare). The decidual leukocytes were further purified by fluorescent activated cell sorting with 7-AAD (420404, Bioledgend) and FITC-labeled anti-CD45 antibody (11-0459-42, eBioscience).

Freshly collected peripheral blood samples were diluted to 1:1 with PBS. Which Peripheral blood after dilution were subjected to lymphocyte enrichment using Ficoll-Paque Plus (17-1440-02, GE Healthcare). The peripheral blood leukocytes were further purified by fluorescent activated cell sorting with 7-AAD (420404, Bioledgend) and FITC-labeled anti-CD45 antibody (11-0459-42, eBioscience).

The freshly purified decidual or peripheral leukocytes were immediately subjected to single cell RNA sequencing as described below.

Generation of single-cell library and transcriptome sequencing

The purified leukocytes from three pairs of normal and RSA cases were separately loaded on Chromium Single Cell Controller (10x Genomics) using the Chromium Single-cell 3' kit v2 to capture 5000 to 8000 cells per sample.

Libraries were sequenced on an Illumina NovaSeq 6000 (Illumina, San Diego) with a read length of 26bp for read 1 (cell barcode and UMI), 8 bp i7 index read (sample barcode), and 98 bp for read 2 (actual RNA read). Reads were first sequenced in the rapid run mode, allowing for fine-tuning sample ratios in the following high-output run. Combining the data from both flow cells yielded approximately > 40,000 reads per cell.

Single-cell RNA-seq data processing and analysis

The raw sequencing reads were processed using Cell Ranger (version 2.0.1, 10x Genomics) (Zheng et al., 2017) The reference index was built using the GRCh38 human reference genome assembly. Cells with fewer than 600 detected genes or with the total mitochondrial gene expression exceeded 5% were removed. Then we convert the obtained matrix into a Seurat object for downstream analysis. All Seurat objects for individual samples were merged into one combined object. According to the integration method reciprocal PCA provided by Seurat, we first performed standard normalization and variable feature selection on each individual sample. Next, we selected features for downstream integration, and ran PCA on each individual sample in the combined object. Since our data include RSA patients and normal pregnant women, we chose one of RSA and normal pregnant women data as the reference, and then used the function FindIntegrationAnchors provided by Seurat to integrate the samples of 6 individuals. Downstream analyses,

including normalization, shared nearest neighbor graph-based clustering, differential expression analysis, and visualization, were performed using the standard workflow provided by Seurat (version 3.0.3). Developmental trajectories were inferred with the Monocle2 (version 2.12.0),. We used the function FindVariableGenes of Seurat to find the variant genes of dNK cells, and then performed trajectory analysis based on the standard workflow provided by Monocle2. We performed differential gene expression analysis of all decidual cell types using Wilcoxon rank sum test inbuilt in Seurat package. differentially expressed genes for dNK clusters were KEGG analysis using R package clusterProfiler(version 3.12.0)(Yu, 2015).

Immunostaining analysis

Human decidual tissues were briefly fixed in 4% PFA and embedded in O.C.T. compound (Sakura Finetek, Torrance, CA, USA). The frozen sections at 10 um were further fixed in 4% PFA and treated with 0.1% triton, and subjected to the incubation with specific antibodies against NCAM1/CD56 (Abcam, ab75813, 1:300), CK7 (Abcam, ab181598, 1:10000), CD39 (proteintech, 14211-1-AP, 1:200), or CD103 (350227, Biolegend, 1:200). Binding of the antibody was visualized using FITC-conjugated or TRITC-conjugated secondary antibody (ZSGB-BIO, ZF-0311, ZF-0313, 1:100), and cell nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma, 28718-90-3, St Louis, MO, USA). The results were recorded using Zeiss LSM780 confocal system (Zeiss,

Oberkochen, BW, Germany) and processed with ZEN 2012 software (Zeiss).
Immunohistochemical staining for Cytokeratin (CK) in decidua was performed
by using antibody against CK7 (ab181598, Abcam, 1:10000) and
HRP-conjugated second antibody (ZSGB-BIO, PV-6001) followed by recovery
of substrate DAB (ZSGB-BIO, ZLI-9019). The images were recorded on a
light microscope with CCD (DP72, Olympus, Japan).

Flow cytometry Assay

Flow cytometry assay for dNK cells was carried out in CytoFLEX (Beckman
Coulter, Inc.) using the following antibodies: PE-labeled anti-CD56(362508,
Bioledgend), Percp-cy5.5-labeled anti-CD3(300328, Bioledgend), APC-labeled
anti-CD39(328209, Bioledgend), APC-cy7-labeled anti-CD103(350227,
Bioledgend), FITC-labeled anti-CD16(302206, Bioledgend), PE-labeled
anti-CD27(356405, Bioledgend), Pacific blue-labeled anti CD11b(301316,
Bioledgend) according to the manufacturer's instructions. Data was analyzed
using CytExpert (Beckman Coulter, Inc.) .

Statistical analysis

Comparison of cell proportion between normal and RSA pregnancies were
statistically analyzed with GraphPad Prism version 7.00 (GraphPad Software,
San Diego, CA, USA). Data were shown as Mean±S.E.M. and comparison
was carried out by unpaired t-test. The P values of less than 0.05 were

considered statistically significant. All the differential expression analyses were performed using the non-parametric two-sided Wilcoxon rank sum test in Seurat.

Data and code availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive(Wang et al., 2017) in National Genomics Data Center(Zhang et al., 2020), Beijing Institute of Genomics (China National Center for Bioinformation), Chinese Academy of Sciences, under accession number HRA000237 that are publicly accessible at <http://bigd.big.ac.cn/gsa-human>.

ACKNOWLEDGEMENTS

We are grateful to Prof. Bin Cao at Xiamen University and Mr. Zhenghui Zhao at IOZ for their critical comments to this study. The technical support from Shiwen Li, Xia Yang and Qing Meng in the experiments of Confocal analysis and FACS was appreciated. We acknowledge all the enrolled patients for their contribution to the study.

This study was supported by grants from the National Key Research and Development Program of China (2018YFC10041002, 2017YFC1001404, 2016YFC1000401, 2016YFC1000200), and the National Natural Science Foundation (81730040, 81490740).

AUTHOR CONTRIBUTIONS

F.W. performed cell isolation, flow cytometry and scRNA sequencing data analysis, and drafted manuscript. W.J. carried out immunostaining and flow cytometry, and participated in data analysis. M.F. was in charge of patient enrollment, sample collection and clinical data checking. Y.L. participated in scRNA sequencing data analysis and manuscript revision. Z.L. helped in immunostaining and flow cytometry. Y.M., X.S. and Y.L. were involved in data analysis and manuscript drafting. Y.L.W., Q.T. and R.L. designed and supervised the study, interpreted the data and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Ander, S.E., Diamond, M.S., and Coyne, C.B. (2019). Immune responses at the maternal-fetal interface. *Sci. Immunol.*
- Arck, P.C., and Hecher, K. (2013). Fetomaternal immune cross-talk and its consequences for maternal and offspring's health. *Nat. Med.*
- Baratin, M., Roetynck, S., Lépolard, C., Falk, C., Sawadogo, S., Uematsu, S., Akira, S., Ryffel, B., Tiraby, J.G., Alexopoulou, L., et al. (2005). Natural killer cell and macrophage cooperation in MyD88-dependent innate responses to *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U. S.*

613 A.

614 Bertrand, L., and Lehuen, A. (2019). MAIT cells in metabolic diseases. *Mol. Metab.*

615 Bulmer, J.N., Morrison, L., Longfellow, M., Ritson, A., and Pace, D. (1991). Granulated

616 lymphocytes in human endometrium: Histochemical and immunohistochemical studies. *Hum.*

617 *Reprod.*

618 Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell

619 transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.*

620 Carlino, C., Stabile, H., Morrone, S., Bulla, R., Soriani, A., Agostinis, C., Bossi, F., Mocci, C.,

621 Sarazani, F., Tedesco, F., et al. (2008). Recruitment of circulating NK cells through decidual

622 tissues: A possible mechanism controlling NK cell accumulation in the uterus during early

623 pregnancy. *Blood.*

624 Committee, P., and Society, A. (2013). Definitions of infertility and recurrent pregnancy loss: A

625 committee opinion. *Fertil. Steril.* 99, 63.

626 Dalbeth, N., Gundle, R., Davies, R.J.O., Lee, Y.C.G., McMichael, A.J., and Callan, M.F.C.

627 (2004). CD56 bright NK Cells Are Enriched at Inflammatory Sites and Can Engage with

628 Monocytes in a Reciprocal Program of Activation . *J. Immunol.*

629 Deshmukh, H., and Way, S.S. (2019). Immunological Basis for Recurrent Fetal Loss and

630 Pregnancy Complications. *Annu. Rev. Pathol. Mech. Dis.*

631 Du, M.R., Wang, S.C., and Li, D.J. (2014). The integrative roles of chemokines at the

632 maternal-fetal interface in early pregnancy. *Cell. Mol. Immunol.*

633 Ebina, Y., Nishino, Y., Deguchi, M., Maesawa, Y., Nakashima, Y., and Yamada, H. (2017).
634 Natural killer cell activity in women with recurrent miscarriage: Etiology and pregnancy
635 outcome. *J. Reprod. Immunol.*

636 Erlebacher, A. (2013). Immunology of the Maternal-Fetal Interface. *Annu. Rev. Immunol.*

637 Fisher, S.J. (2015). Why is placentation abnormal in preeclampsia? *Am. J. Obstet. Gynecol.*
638 213, S115–S122.

639 Fridman, W.H., Pagès, F., Saúts-Fridman, C., and Galon, J. (2012). The immune contexture in
640 human tumours: Impact on clinical outcome. *Nat. Rev. Cancer.*

641 Fu, B., Wang, F., Sun, R., Ling, B., Tian, Z., and Wei, H. (2011). CD11b and CD27 reflect
642 distinct population and functional specialization in human natural killer cells. *Immunology.*

643 Fu, B., Li, X., Sun, R., Tong, X., Ling, B., Tian, Z., and Wei, H. (2013). Natural killer cells
644 promote immune tolerance by regulating inflammatory TH17 cells at the human maternal-fetal
645 interface. *Proc. Natl. Acad. Sci. U. S. A.*

646 Gamliel, M., Goldman-Wohl, D., Isaacson, B., Gur, C., Stein, N., Yamin, R., Berger, M.,
647 Grunewald, M., Keshet, E., Rais, Y., et al. (2018a). Trained Memory of Human Uterine NK
648 Cells Enhances Their Function in Subsequent Pregnancies. *Immunity* 48, 951-962.e5.

649 Gamliel, M., Goldman-Wohl, D., Isaacson, B., Gur, C., Stein, N., Yamin, R., Berger, M.,
650 Grunewald, M., Keshet, E., Rais, Y., et al. (2018b). Trained Memory of Human Uterine NK
651 Cells Enhances Their Function in Subsequent Pregnancies. *Immunity.*

652 Gentek, R., Molawi, K., and Sieweke, M.H. (2014). Tissue macrophage identity and

653 self-renewal. *Immunol. Rev.*

654 Godfrey, D.I., Koay, H.F., McCluskey, J., and Gherardin, N.A. (2019). The biology and

655 functional importance of MAIT cells. *Nat. Immunol.*

656 Haghverdi, L., Lun, A.T.L., Morgan, M.D., and Marioni, J.C. (2018). Batch effects in single-cell

657 RNA-sequencing data are corrected by matching mutual nearest neighbors. *Nat. Biotechnol.*

658 Hanna, J., Wald, O., Goldman-Wohl, D., Prus, D., Markel, G., Gazit, R., Katz, G.,

659 Haimov-Kochman, R., Fujii, N., Yagel, S., et al. (2003). CXCL12 expression by invasive

660 trophoblasts induces the specific migration of CD16⁺ human natural killer cells. *Blood.*

661 Hanna, J., Goldman-Wohl, D., Hamani, Y., Avraham, I., Greenfield, C., Natanson-Yaron, S.,

662 Prus, D., Cohen-Daniel, L., Arnon, T.I., Manaster, I., et al. (2006). Decidual NK cells regulate

663 key developmental processes at the human fetal-maternal interface. *Nat. Med.*

664 Huhn, O., Ivarsson, M.A., Gardner, L., Hollinshead, M., Stinchcombe, J.C., Chen, P., Shreeve,

665 N., Chazara, O., Farrell, L.E., Theorell, J., et al. (2020). Distinctive phenotypes and functions

666 of innate lymphoid cells in human decidua during early pregnancy. *Nat. Commun.*

667 Kheshtchin, N., Gharagozloo, M., Andalib, A., Ghahiri, A., Maracy, M.R., and Rezaei, A. (2010).

668 The expression of Th1- and Th2-related chemokine receptors in women with recurrent

669 miscarriage: The impact of lymphocyte immunotherapy. *Am. J. Reprod. Immunol.*

670 King, A., Balendran, N., Wooding, P., and Loke, Y.W. (1991). Cd3⁺ Leukocytes Present in the

671 Human Uterus During Early Placentation: Phenotypic and Morphologic Characterization of the

672 Cd56⁺⁺ Population. *Dev. Immunol.*

673 King, K., Smith, S., Chapman, M., and Sacks, G. (2010). Detailed analysis of peripheral blood
674 natural killer (NK) cells in women with recurrent miscarriage. *Hum. Reprod.*

675 Klentzeris, L.D., Bulmer, J.N., Warren, M.A., Morrison, L., Li, T.C., and Cooke, I.D. (1994).
676 Lymphoid tissue in the endometrium of women with unexplained infertility: Morphometric and
677 immunohistochemical aspects. *Hum. Reprod.*

678 Kuon, R.J., Vomstein, K., Weber, M., Müller, F., Seitz, C., Wallwiener, S., Strowitzki, T.,
679 Schleussner, E., Markert, U.R., Daniel, V., et al. (2017). The “killer cell story” in recurrent
680 miscarriage: Association between activated peripheral lymphocytes and uterine natural killer
681 cells. *J. Reprod. Immunol.*

682 Lachapelle, M.H., Miron, P., Hemmings, R., and Roy, D.C. (1996). Endometrial T, B, and NK
683 cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. *J.*
684 *Immunol.*

685 Ma, L., Li, G., Cao, G., Zhu, Y., Du, M.R., Zhao, Y., Wang, H., Liu, Y., Yang, Y., Li, Y.X., et al.
686 (2017). DNK cells facilitate the interaction between trophoblastic and endothelial cells via
687 VEGF-C and HGF. *Immunol. Cell Biol.*

688 Manaster, I., Mizrahi, S., Goldman-Wohl, D., Sela, H.Y., Stern-Ginossar, N., Lankry, D., Gruda,
689 R., Hurwitz, A., Bdolah, Y., Haimov-Kochman, R., et al. (2008). Endometrial NK Cells Are
690 Special Immature Cells That Await Pregnancy. *J. Immunol.*

691 Nancy, P., Tagliani, E., Tay, C.S., Asp, P., Levy, D.E., and Erlebacher, A. (2012). Chemokine
692 gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface.
693 *Science*7.

694 Parham, P., and Moffett, A. (2013). Variable NK cell receptors and their MHC class I ligands in
695 immunity, reproduction and human evolution. *Nat. Rev. Immunol.*

696 PrabhuDas, M., Bonney, E., Caron, K., Dey, S., Erlebacher, A., Fazleabas, A., Fisher, S.,
697 Golos, T., Matzuk, M., McCune, J.M., et al. (2015). Immune mechanisms at the maternal-fetal
698 interface: Perspectives and challenges. *Nat. Immunol.*

699 Seshadri, S., and Sunkara, S.K. (2014). Natural killer cells in female infertility and recurrent
700 miscarriage: A systematic review and meta-analysis. *Hum. Reprod. Update.*

701 Sotnikova, N., Voronin, D., Antsiferova, Y., and Bukina, E. (2014). Interaction of decidual
702 CD56+ NK with trophoblast cells during normal pregnancy and recurrent spontaneous abortion
703 at early term of Gestation. *Scand. J. Immunol.*

704 Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., Hao, Y.,
705 Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell
706 Data. *Cell.*

707 Suryawanshi, H., Morozov, P., Straus, A., Sahasrabudhe, N., Max, K.E.A., Garzia, A., Kustagi,
708 M., Tuschl, T., and Williams, Z. (2018). A single-cell survey of the human first-trimester
709 placenta and decidua. *Sci. Adv.*

710 Vacca, P., Vitale, C., Montaldo, E., Conte, R., Cantoni, C., Fulcheri, E., Darretta, V., Moretta,
711 L., and Mingari, M.C. (2011). CD34+ hematopoietic precursors are present in human decidua
712 and differentiate into natural killer cells upon interaction with stromal cells. *Proc. Natl. Acad.*
713 *Sci. U. S. A.*

714 Varol, C., Mildner, A., and Jung, S. (2015). Macrophages: Development and Tissue

715 Specialization. *Annu. Rev. Immunol.*

716 Vento-Tormo, R., Efremova, M., Botting, R.A., Turco, M.Y., Vento-Tormo, M., Meyer, K.B.,
717 Park, J.E., Stephenson, E., Polański, K., Goncalves, A., et al. (2018). Single-cell
718 reconstruction of the early maternal–fetal interface in humans. *Nature.*

719 Wallace, A.E., Host, A.J., Whitley, G.S., and Cartwright, J.E. (2013). Decidual natural killer cell
720 interactions with trophoblasts are impaired in pregnancies at increased risk of preeclampsia.
721 *Am. J. Pathol.*

722 Wang, F., Zhou, Y., Fu, B., Wu, Y., Zhang, R., Sun, R., Tian, Z., and Wei, H. (2014). Molecular
723 signatures and transcriptional regulatory networks of human immature decidual NK and
724 mature peripheral NK cells. *Eur. J. Immunol.* *44*, 2771–2784.

725 Wang, W.J., Hao, C.F., Yi-Lin, Yin, G.J., Bao, S.H., Qiu, L.H., and Lin, Q. De (2010). Increased
726 prevalence of T helper 17 (Th17) cells in peripheral blood and decidua in unexplained
727 recurrent spontaneous abortion patients. *J. Reprod. Immunol.*

728 Wang, Y., Song, F., Zhu, J., Zhang, S., Yang, Y., Chen, T., Tang, B., Dong, L., Ding, N., Zhang,
729 Q., et al. (2017). GSA: Genome Sequence Archive*. *Genomics, Proteomics Bioinforma.* *15*,
730 14–18.

731 Wu, X., Jin, L.-P., Yuan, M.-M., Zhu, Y., Wang, M.-Y., and Li, D.-J. (2005). Human
732 First-Trimester Trophoblast Cells Recruit CD56 bright CD16 – NK Cells into Decidua by Way
733 of Expressing and Secreting of CXCL12/Stromal Cell-Derived Factor 1 . *J. Immunol.*

734 Yang, F., Zheng, Q., and Jin, L. (2019). Dynamic Function and Composition Changes of
735 Immune Cells During Normal and Pathological Pregnancy at the Maternal-Fetal Interface.

736 Front. Immunol.

737 Yang, H., Qiu, L., Chen, G., Ye, Z., Lü, C., and Lin, Q. (2008). Proportional change of

738 CD4+CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent

739 spontaneous abortion patients. Fertil. Steril.

740 Yu, G. (2015). Statistical analysis and visualization of functional profiles for gene and gene

741 clusters. Bioconductor.Org.

742 Zhang, J., Dunk, C., Croy, A.B., and Lye, S.J. (2016). To serve and to protect: the role of

743 decidual innate immune cells on human pregnancy. Cell Tissue Res.

744 Zhang, Z., Zhao, W., Xiao, J., Bao, Y., He, S., Zhang, G., Li, Y., Zhao, G., Chen, R., Gao, Y., et

745 al. (2020). Database Resources of the National Genomics Data Center in 2020. Nucleic Acids

746 Res.

747 Zheng, G.X.Y., Terry, J.M., Belgrader, P., Ryvkin, P., Bent, Z.W., Wilson, R., Ziraldo, S.B.,

748 Wheeler, T.D., McDermott, G.P., Zhu, J., et al. (2017). Massively parallel digital transcriptional

749 profiling of single cells. Nat. Commun.

750 Zhou, Y., Fu, B., Xu, X., Zhang, J., Tong, X., Wang, Y., Dong, Z., Zhang, X., Shen, N., Zhai, Y.,

751 et al. (2020). PBX1 expression in uterine natural killer cells drives fetal growth. Sci. Transl.

752 Med.

753

754 **FIGURE LEGENDS**

755

756 Fig. 1. Landscape of immune cells from peripheral blood and decidual tissues in normal

pregnant women and RSA patients at early pregnancy using single-cell RNA-seq.

(A) UMAP plot of scRNA-seq data to show the 22 leukocyte clusters in peripheral blood and decidual tissues at early pregnancy. (B) UMAP plot showing cell populations from peripheral blood and decidual tissue. (C) UMAP visualization of cell clustering in normal and RSA pregnancies. (D) Characteristics of the 22 leukocyte clusters based on canonical marker genes expression for cell type annotation.

Fig. 2. Characteristics of the peripheral blood leukocyte clusters indicate the maternal immune inflammatory status in RSA patients.

(A) Heatmap of the top5 genes expressed in peripheral blood T cell subsets. x-axis represents T cell clusters; y-axis represents genes. (B) Box plots depicting cell composition of peripheral blood T cells in healthy control and RSA patients. (C) Violin plots of selected markers identified by differential expression analysis comparing the MAIT subset between RSA and CTRL patients. (D) UMAP visualization of the log-transformed, normalized IFNG expression showing the relatively stronger cytotoxicity of CD8 T effector in RSA pregnancy. high expression is showed in red, low expression is illustrated in grey. (E) Box plots depicting cell number ratio of peripheral NK cell subsets in peripheral blood of normal and RSA pregnancies. (F) Violin plots of indicated genes identified by differential expression analysis of NK dim subset between RSA and CTRL patients. Differential expression analyses in Fig. 2 were performed using the non-parametric two-sided Wilcoxon rank sum test in Seurat.

Fig. 3. Characteristics of decidual leukocyte subpopulations demonstrated an immune activation status at the maternal-fetal interface of RSA pregnancy.

(A) Cell proportion of T, dM and dNK cell subsets in decidual tissues of normal and RSA pregnancies. (B) and (C) Analysis of the ligand-receptor interactions among the leukocyte subpopulations between RSA and normal pregnancies in decidua. C: CTRL, R: RSA. (D) Volcano plot showing globally differential genes in decidual macrophages between normal and RSA pregnancies. (E) Expression of anti-inflammation and inflammation -associated cytokines in decidual macrophage subpopulation in normal and RSA pregnancies. Differential expression analyses in D were performed using the non-parametric two-sided Wilcoxon rank sum test in Seurat. Panels

Fig. 4. Molecular aspects and developmental trajectories of human dNK subtypes

(A) Heatmap showing relative expression (z-score) of 20 marker genes defining the subpopulations of dNK cells. (B) Functional KEGG enrichment analysis illustrating the functional signature of the dNK subpopulations. (C) Expression of KIR molecules mediating in the indicated dNK subpopulations. (D) Flow cytometry analysis showing the dNKc cells do not express CD27 and CD11b, further demonstrating their low-differentiation status. (E) Monocle2 analysis showing the developmental trajectories of dNK cells. (F) The expression of classical transcription factors in dNK subpopulations indicating their different developmental status.

Fig. 5. Immunofluorescence to show the spatial distribution of dNK cells at the

feto-maternal interface

(A) Immunohistochemistry for cytokeratin 7 (CK7) was used to locate trophoblast and uterine gland epithelium in the sections. (B) and (C) Immunofluorescence staining show the spatial distribution of dNKc (CD39+) and dNKd (CD103+) cells at the feto-maternal interface at early pregnancy. Scale bar in panel B and C from left to right are 100, 25, 10 μ m. (D) The spatial distribution of dNKe in decidual tissues from normal and RSA pregnancies by Immunofluorescence staining. Scale bar of panel D, 25 μ m. The arrow marks the target cell we zoomed in Fig. 5 B, C and D. The dotted line in panel D indicates the position of blood vessels

Fig. 6. Alterations of dNK subsets in decidua of RSA pregnancy

(A) Flow cytometry to sort dNKc (CD39+), dNKd (CD103+) and dNKe(CD16+) cells in human decidual tissues. (B) Cell population of dNKc and dNKd in decidua tissues from normal pregnancy (n=5) and RSA (n=5). Lower panel, Statistical results showing cell proportions of dNKe in decidua tissues from normal pregnancy (n=4) and RSA (n=11). (C) Violin plots revealing differential expression of indicated genes in dNKc, dNKd and dNKe subsets from RSA and CTRL patients. Significance was analyzed using unpaired t-tests assuming no consistent s.d..

Fig. S1. Gating strategy and quality control for droplet single-cell RNA-seq.

(A) Flow Sorting of living leukocytes from peripheral blood and decidual tissue using CD45 antibody and 7AAD. (B) Workflow to depict the dissociation and sorting of CD45+

leukocytes from peripheral blood and decidual to generate scRNA transcriptome profile.

(C) Total number of cells that met the requirement of quality control and subjected to droplet scRNA-seq. (D) Quality evaluation on sequencing data including nFeature_RNA and nCount_RNA in peripheral blood and decidua. E, Every cluster distribution of each cell subpopulation in the 6 samples.

Fig. S2. The MNN algorithm to confirm the appropriate elimination of the batch effect of the data.

(A) Data from the 6 samples were integrated with MNN and visualized using UMAP. (B) Distribution of leukocytes from peripheral blood and decidual tissues after MNN integration. (C) Mutual nearest neighbors (mnn_1 and mnn_2) of the integrated analysis of decidual and peripheral blood leukocytes from the droplet-based datasets. (D) Distribution of leukocytes from normal pregnancy and RSA after MNN integration.

Fig. S3. Identification of peripheral blood T cell subpopulations by specific marker genes.

(A) UMAP visualization of peripheral blood T cells. (B)-(E), Feature plot of Naïve T (B), MAIT T (C), CD4T memory (D) and CD8T effector (E) cells with specific marker genes.

Fig. S4. Top100 genes in dNK cells that change in a continuous manner over pseudotime

(A) Heatmap showing the top100 genes in dNK cells that change in a continuous manner over pseudotime

845 Fig. S5. Globally differential genes in CD16+ NK cell between dNKe from decidua and NK

846 dim from peripheral blood

847 (A) Volcano plot shows that upregulated and downregulated genes between dNKe and

848 NK dim. Differential expression analyses in dNKe were performed using the

849 non-parametric two-sided Wilcoxon rank sum test in Seurat.

850

851 Fig. S6. Identification of decidual macrophage subpopulations

852 (A) UMAP showing two subpopulations of decidual macrophage. (B) UMAP showing

853 macrophage subpopulations in normal pregnancy (grey) and RSA (red). (C)

854 Characterization of the two macrophage subpopulations using marker genes.

855

A Fig. 1 **B** **C** **D**

Fig. 2

Fig. 5

Fig. 6

A

B

C